IDENTIFICATION AND LOCALIZATION OF CARDIAC MYOSIN IN PREIMPLANTATION AND CULTURED RABBIT EMBRYOS

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ABSTRACT

BIOLOGY

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Identification and Localization of Cardiac Myosin in the Preimplantation and Cultured Rabbit Embryo.

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Early identification and localization of pre-cardiac cells in the embryonic development of mammals, specifically during the blastocyst stage, has not been studied nearly as completely as in other classes. Because of the requirements of the developing embryo, the heart starts its definitive function early in development. Myosin, one of the major contractile proteins in muscle tissue, is required.

The purpose of this research was to evaluate and investigate the ontogeny of cardiac myosin as a marker for differentiated heart tissue. The study was undertaken to identify and localize precardiac cells using cardiac myosin as a marker in preimplantation rabbit embryos. Guinea pigs were injected with both embryonic and adult cardiac myosin and the antibodies produced were immunologically tested. The approach involves SDS-polyacrylamide gel electrophoresis, agar-gel diffusion, immunoelectrophoresis, quantitative immunoprecipitation, histology, in vitro studies and immunofluorescence (microscope slide and cover slip method) using conjugated fluorescein isothiocyanate labelling techniques. Cardiac myosin has two heavy chains approximately 200,000 D and two light chains LC-1 25,000 D and LC-2 17,500 D

and a C-protein 40,000 D. Isolated cardiac myosin cross-reacted with antibodies prepared against both adult and embryonic myosins. Embryonic discs were removed and cultured in Petri dishes alone or in Petri dishes containing cover slips. After two days in culture, the embryonic disc attaches to the bottom of the Petri dish or to the cover slip. After three days in culture pulsation begins. Pulsation and growth (differentiation) proceed in an orderly fashion which indicates the necessity of sequential induction for progressive development.

Using the microscope slide technique, myosin was localized in the 6 3/4-day-old embryonic disc and in the 10-day-old embryonic heart. The cover slip fluoresence technique showed that myosin was localized in the cultured 6, 6 3/4, 7 3/4 and 8 3/4-day-old embryonic disc cells, but not in the 5 3/4-day-old embryonic disc cells. The distribution of fluorescence increased with increasing age of the embryo. No indications of pre-cardiac cells were found in the trophoblast or in the liver control slides.

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CHAPTER I

INTRODUCTION

Studies of mammalian development present particular difficulties as compared with the study of the development of egg-laying animals. In spite of the particular interest that mammalian development has for man, studies on mammalian eggs and embryos have lagged behind those made on lower animals. This applies to an even greater degree to the study of the embryonic development of man. In other mammals, eggs and embryos can be obtained by sacrificing fertilized and pregnant females, but in humans, investigators are limited to accidental findings obtained as a result of operations or autopsies on women.

One approach to studying mammalian development is to obtain newly fertilized eggs and to study their subsequent development. This is done by knowing when ovulation and fertilization have taken place. In humans, the levels of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) determine ovulation. In some mammals, the time of ovulation is determined by the occurrence of coitus. The release of the FSH and LH depends on the nervous excitation accompanying coitus. The rabbit is one mammal in which this mechanism is present, and for this reason the rabbit has become one of the favorite animals for the study of mammalian development. Ovulation in the rabbit occurs ten and one half (10.5) hours after coitus. Embryos can be collected easily up to 6 3/4 days of development.

The fertilized rabbit egg develops into an embryo which consists of a trophoblast and an embryonic disc. Blastulation begins in the rabbit about three and one half (3.5) days post coitus (p.c.). Daniel (1964) discovered that swelling of the spherical mass in some way separates whole cells that will form the trophoblast from the inner cell mass. There is a rapid expansion of the blastocyst from 3 1/2 to 7 days. It is believed that the expansion is due to the accumulation of fluid within the blastocoel. At 6 3/4 days, the blastocyst consists of two morphologically distinct areas,the trophoblast and the inner cell mass. The inner cell mass will develop into the embryo proper.

The heart in meroblastic vertebrates develops before the body of the embryo becomes separated from the yolk sac (DeHaan, 1967). The embryo at this point is still lying flat on the surface of the yolk. Of all the organs of a vertebrate, the heart is the one which starts its definitive function earliest (DeHaan, 1967). The embryo is also greatly dependent in its development on the heart's function. The major contractile protein, specifically myosin, must be present for the heart to contract.

The developing embryonic mammalian heart has not been studied nearly as completely as the avian heart. Hopefully, informative morphological parameters of the avian heart studies will coincide with our studies dealing with the embryonic mammalian heart. Cahn (1964), and Kuramitsu and Harary (1964) have shown biochemically that cultured beating chick and rat heart cells behave differently from beating cells in the intact organs.

We have observed in our laboratory that when fragmented blastocysts or trophoblast-free embryonic discs are cultured, beating masses form in 3 days. According to Finkelstein et al. (1976) in a period of ninety-six (96) hours, a progenitor cell gives rise to thousands of cells that are capable of contracting. The contractile cells undergo morphogenetic movements to form a large synchronous mass. DeHaan (1968) has maintained beating cell masses from chick embryos for three to four weeks. In vitro studies of pulsating cells offer an excellent opportunity to study a variety of the different parameters of differentiation, as well as organogenesis.

Holtzer et al. (1972) recognized that the core problem of cell differentiation is neither the identification of the inducing molecules, nor the determination of the de novo biochemical pathways elicited by the inducing molecules. The central problem of differentiation pertains to those mechanisms that make available genetic information in daughter cells that was not readily available in the mother cell. However, one realizes that there is a distinction between cell differentiation and cell determination. Cell determination is the process which limits cell potential during development. Holtzer (1972) feels that to say a cell in the blastocyst stage is "determined" to become a muscle cell is false. More probable, the first event establishing "myogenic" linkage is followed by a second and a third event in subsequent generations until a generation emerges that has accumulated the necessary machinery to produce all those molecular characteristics of fully differentiated muscle.

Although a considerable amount of information has been obtained pertaining to mammalian heart cells, a tremendous amount of information which involves differentiation still remains unsolved. The goals of this research project are to investigate and evaluate the ontogeny of cardiac myosin and differentiated heart development. This study is undertaken to determine if the presence of a specific heart protein, myosin, can be detected early enough in embryonic development to be used as a marker for the identification of precardiac cells in rabbit embryos.

The approach involves SDS-polyacrylamide gel electrophoresis, agar-gel diffusion, immunoelectrophoresis, quantitative immunoprecipitation, histology, *in vitro* studies and immunofluoresence.

CHAPTER II

REVIEW OF LITERATURE

A. Isolation Of Cardiac Myosin from Adult Rabbit Heart

Efforts to characterize the structural and functional properties of cardiac myosin in different pathophysiological conditions are often impaired by an inability to isolate sufficient yields of high purity myosin. In the past two decades considerable information has been obtained with respect to the structure and enzymatic properties of myosin from *in vitro* studies. However, the bulk of this information concerns rabbit skeletal myosin (Szent-Gyorgyi, 1951). The difficulty of purifying cardiac myosin (Katz, 1970) in conjunction with its tendency towards aggregation, has discouraged, somewhat, an equally extensive investigation of this important contractile protein. Because of the limited availability of fresh rabbit hearts in the past, Brahms and Kay (1963), Mueller et al. (1964), Tada et al. (1969), and Vierling et al. (1968) chose to study cardiac myosin by preparing it from canine or bovine tissue. Consequently, little research has been directed towards rabbit cardiac myosin, and its complete characterization has not been carried out.

A difficulty frequently encountered in the purification of myosin is the removal of actomyosin, tropomyosin and troponin. The procedure of Hasselback and Schneider (1951) employs prolonged extraction, tissue homogenization and stirring to promote myosin extraction from muscle. Unfortunately, this procedure also promotes concurrent removal of actomyosin. Other investigators have reported frequent contaminants in myosin preparations, which include RNA (Baril et al., 1966 and Gaetjens et al., 1968), myokinase and AMP deaminase (Richards et al., 1967). Harris and Seulter (1967) discovered that the chromatographic techniques or $(NH_4)_2SO_4$ fractionation by Luchii et al. (1965) and Wikman-Coffelt et al. (1973) remove contaminants but substantially reduce the yield of cardiac myosin (Swynghedauw et al., 1973 and Kleid et al., 1972).

Myosin, a globular protein from muscle cells, generally consists of two identical heavy chains (approximately 200,000 D each) and two different light chains (15,000 D - 27,000 D). To understand fully the molecular events of muscle contraction and cell mobility, it is necessary to evaluate the roles of each of the myosin subunits by the hydrolysis of ATP by myosin. The myosin monomer is grossly asymmetric, with the amino-terminal portion of the molecule being globular in shape and the carboxyl-terminal portion being fibrous. The globular part of the molecule contains a binding site for actin as well as a catalytic site that hydrolyzes ATP.

Muscle contraction consists of the attachment of the globular portion of the myosin molecule to the actin filament followed by a major change in the angle of myosin-actin attachment, so that the myosin and actin filaments slide past each other (Huxley, 1969, 1974). The energy for this physical alteration is provided by ATP and is released by the interaction of actin with myosin.

Regulation of muscle contraction involves modifying the interaction of actin and myosin. This can be accomplished by interaction of actoymosin with other proteins such as the complex of troponin-tropomyosin, which in skeletal and cardiac muscle causes the actin-activated ATPase activity to be dependent on 10^{-5} M Ca^{+2} ions (Weber and Murray 1973). A second form of regulation, only recently found to be present in muscles, is by reversible covalent phosphorylation of the proteins involved in the contraction-relaxation process (Perry 1979).

The procedure used to extract cardiac myosin is that of Shiverick et al. (1974) modified by Broadway (1978), and incorporates steps from the method of Baril et al. (1966) for embryonic skeletal muscle with the basic multiple precipitation procedure of Mueller et al. (1964) for cardiac muscle.

B. Isolation of Cytoplasmic Myosin from Adult Rabbit Liver

Most of the knowledge of the properties and structure of myosin has been obtained with skeletal muscle. Relatively little information is available concerning myosin isolated from smooth muscle.

The localization of myosin in non-muscle cells is essential for elucidating its possible functions in cell mobility. Since myosin molecules can not be visualized directly in non-muscle cells by electron microscopy, several laboratories have attempted to localize myosin in nonmuscle systems using immunological techniques.

Myosin-like proteins have been found in many non-muscle cells and tissues including Amoebae (Pollard and Korn, 1973); Dictyostelium (Clarke and Spudich, 1974); platelets (Bettex-Galland and Luscher, 1959; Adelstein et al., 1971); leukocytes (Stossel and Pollard, 1973), brain (Berl et al., 1973; Burridge and Bary, 1975); liver, (Brandon, 1976); gizzard (Barany et al., 1966) and several others. On the basis of sodium dodecyl sulfate gel densitometry and ATPase measurements, Hartwig and Stossel, 1975; Ostlund and Pastan, 1976; and Yerna et al. 1978 concluded that myosin represents 0.3-5.0% of the total cell protein in non-muscle systems.

At low ionic strength, (Kobayashi et al., 1977 and Shibata et al., 1977) mammalian non-muscle myosin molecules aggregate into synthetic bipolar thick filaments similar in shape to, but shorter than, muscle filaments formed under analogous conditions.

Pollard and Weihing, (1974) and Goldman et al. (1976) suggested that cytoplasmic myosin is involved in various aspects of motility. In 1974, Pollard et al. concluded, based on molecular weight, that smooth muscle myosin resembled striated muscle myosin with two different heavy chains and two or three light chains (depending on the animal species) one of which is not essential for ATPase activity (Weeds and Frank, 1973). This difference in light chains is not unexpected. It has been shown by amino acid sequencing that one of the rabbit cardiac myosin light chains is unrelated to the skeletal muscle myosin light chains from the same animal (Weeds and Frank, 1973). It would be interesting if the amino acid sequence in the heavy chains of the cytoplasmic myosin were the same as the cardiac and skeletal muscle myosin heavy chain sequence.

C. Electrophoresis

The separation of serum protein by an electric field (electrophoresis) depends on the net charge of the protein molecules and to a lesser extent on their size. Svedberg and Tiselius (1962) are considered as pioneers in the area of electrophoresis. Hunter and Burstone (1958) used Smithies' (1955) zone electrophoresis method to characterize enzyme substrate specificity. Zone electrophoresis provides a comparatively innocuous method for the separation of ionic mixtures and is based on differences among the electrophoretic mobilities of the constituent ions. The development of zone electrophoresis in starch gel matrices allows for the capacity of gels to "sieve" high molecular weight substances such as proteins. Starch gel electrophoresis is carried out in discontinuous buffer (Poulik, 1957).

In 1971, Neville reported methods and results obtained by combining the techniques of sodium dodecyl sulfate (SDS) gel electrophoresis and electrophoresis in discontinuous buffer systems. According to Neville, the SDS gel system utilizes a sulfate-borate discontinuity, which stacks and unstacks protein - SDS complexes over a range of 2,300 to 320,000 daltons, providing high resolution fractionation.

One-dimension (1-D) SDS-polyacrylamide disc gel electrophoresis (separating proteins based on molecular weight) has been done in vertical glass tubes and on slabs. Two-dimensional (2-D) gel electrophoresis, involves the separation of proteins by both tube (according to charge by isoelectric focusing) and slab (on the basis of molecular weight). Due to its resolution and sensitivity, Ames and Nikaido (1976) used two-dimensional gel electrophoresis for separation of proteins from complex biological sources.

D. Antigen-Antibody Reaction

1. Ouchterlony Method: Ouchterlony (1948), developed the most useful technique for characterizing complex relationships between an antigen and antibody by gel "double diffusion" in two-dimension. This method requires a layer of agar gel deposited in a Petri dish with circular wells made near one another in the gel. An antibody is added to the center well with antigens placed in the outer wells. The materials are allowed to diffuse. As the material diffuses, the concentration of the substance decreases. When an optimal precipitating ratio of the counter-diffusing materials is reached in this zone, a line of precipitation is formed in the gel.

A three-well Ouchterlony method is particularly useful for comparing antigens or antisera for the presence of identical, partially identical or non-cross-reacting components.

2. Immunoelectrophoresis: Immunoelectrophoresis (IEP) is a process by which serum proteins are first separated by electrophoresis and then allowed to react by diffusion with a particular polyvalent or monospecific antiserum. The technique of IEP was introduced in 1953 by Grabar and Williams and later modified by Scheidegger (1955) into a micro-method which proves to be extremely adaptable to routine clinical use. To identify specific protein abnormalities in serum samples, IEP combines the separation capabilities of zone electrophoresis with the very specific detection capability of the antigen-antibody precipitin reaction.

In diverse research situations, IEP is used to enumerate antigenic components in mixtures and to monitor antigen and antibody purification. It is used to analyze soluble tissue antigens of plants and animals and antigens in extracts of microorganisms and culture media. Immunoelectrophoresis can also be used to study growth, development and biosynthesis, as well as to detect genetic variance due to mobility differences of blood serum proteins and microbial extracts (Williams and Chase, 1973).

3. Precipitin Reaction: Under appropriate experimental conditions, including the chemical nature of the antigen, antigen-antibody complexes precipitate out of solution. Since antibody molecules are bivalent, that is, they contain two antigen binding sites, antibodies when complexed with antigens, can form a crosslinked mass.

Arquembourg et al. (1970) states that antigen-antibody precipitin reactions are the result of several attracting forces. These forces include atomic dispersion (van der Waals) forces, hydrogen bonding, dipole interaction, electrostatic attraction and attraction between nonpolar antigen and antibody surfaces.

Beginning work in the quantitative investigation of precipitin reaction dates from the experiments conducted by Heidelberger and Kendall (1929). He first defined these reactions in a fluid medium by the nitrogen analysis of the formed precipitate. These quantitative methods were later modified by Boyden (1974), whose procedure for working in a liquid medium included the photometric analysis of the precipitated antigen-antibody complexes. Conveniently, a precipitin reaction can also occur in a gel medium whereby visual inspection of the precipitates allows for detection of the protein being analyzed.

4. Immunoprecipitation: Immunoprecipitation is the process by

which an antigen and an antibody form cross-bridges and precipitate out in solution. Several years ago investigators began using a two-step indirect method described by Kessler (1975) for mammalian membrane proteins, in which immune complexes are bound to Protein A on the bacterial cell wall of formalin-fixed, heat-inactivated *Staphylococcus areus* Cowan Type I. The immune complexes bound were isolated from solution by centrifugation, washing, and elution with denaturing reagents. In 1981, Goldman and Blobel used immunoprecipitation to assay for specific protein synthesis of a membrane-integrated, core glycosylated form of bovine opsin.

5. Immunofluorescence: Immunofluorescence microscopy has been used in recent years to identify and localize contractile proteins in a wide variety of cell types, both muscle and nonmuscle. In 1933, Hopkins and Wormall were the first to conjugate proteins to aromatic isocyanates by carbamido linkage. The most likely reactive site is believed to be the -amino group of lysine. Conjugated isocyanates of higher aromatic hydrocarbons to protein was worked out by Creech and Jones (1941). Using fresh-frozen or dry-frozen tissue, Coons and Kaplan (1950), Coons et al. (1951), and Marshall (1951) showed that fluorescent-labelled antibodies may identify antigen localized in the tissue. Using a well-defined antigen, Clayton and Feldman's (1955) method yields results resembling those of Coon's method. Immunofluorescent studies have contributed an abundance of information in all areas of research.

In 1953 Elbert, using light microscopy techniques detected cardiac myosin in the chick's primitive streak before any indication of the differentiated heart was observed. Gordon and Bushnell (1979) examined microfilament networks in re-spreading non-muscle cells using immunofluorescence studies. Based on the timing of their appearance and disappearance in re-spreaded rat embryo cells, they concluded that polygonal networks may serve as structural precursors of stress fibers. Connally et al. (1978) used immunofluorescence to localize intracellular microtubular proteins. Bagby (1980) and Campbell et al. (1979) have been applying this method to the study of smooth muscle cells in culture in an attempt to gain further information on the organization of contractile and cytoskeletal elements in smooth muscle grown _in vitro_ under normal and pathological conditions.

Fluorescent antibody staining techniques have been used successfully in conjunction with electron microscopy to gain additional information about the location of proteins in skeletal muscle myofibrils (Pepe, 1975 and Offer, 1976), and have been used extensively in studies of the organization of contractile proteins in various types of cultured cells (Lazaides, 1975, 1976; Goldman et al., 1975).

E. _Early Organogenesis_

The development of an organ, or a multicellular organism, represents the synthesis of three component processes. These are growth, differentiation and morphogenesis. Growth is an increase in size resulting from mitotic activity, that is, increase in cell number. The increase in cell number requires the synthesis of structural proteins and of all the other different molecules that are present in a cell. Differentiation is the appearance of new characteristics in cells, including new behavioral properties, new compounds and new cell structures which did not exist in the cell or in its progenitors at an earlier stage. Morphogenesis is the capacity of cells to move, to adhere to one another, to exhibit membrane activity, to change shape and thus to aggregate into condensed cell masses or cohesive sheets which can be molded into functional tissues and organs.

Although a considerable amount of work has been done concerning embryonic development following fertilization of the egg to early stages of the blastocyst, little has been done concerning embryonic development from the blastocyst to early implantation stages. Cole and Paul (1965) observed the formation of beating myocardial cells, muscle cell types, blood islands and nerve cells in culture. They stated that if the zona pellucida was removed by pronase, an embryo would form, the amniotic folds would close and the beating heart would develop.

The heart of the rabbit embryo like the heart of all vertebrates is composed of a network of striated muscle fibers which contract rhythmically and spontaneously. Cardiac muscle consists of striated muscle fibers which differ in several respects from those of skeletal muscle. The fibers are not syncytial, but are made up of separate cellular units joined end to end by special surface membrane specializations, intercalated discs, that run transversely across the fiber (Maximow and Bloom, 1957). Although the significance of the intercalated discs is unknown, Lesson and Lesson (1976) suggest that the intercalated discs act as fine, elastic interstitial tendons.

Intercalated discs have also been considered to be thickenings which divide the network of cardiac muscle fibers into cellular territories. This view is supported by the electron microscopic study of Sjostrand et al. (1958). The intercalated disc appear comparatively late in the development of cardiac muscle and their number gradually increase with age, independent of cell multiplication (Maximow and Bloom, 1957).

Chick embryos have been cultured _in vitro_ during the time the heart was forming, under circumstances which provided good optical viewing conditions (Dehaan, 1968). It was possible to trace the movement of individual cells or cell masses as the heart formed under these conditions because of the extended time required for the events to occur. Developmental events were condensed into a period of minutes using time lapse cinematography. Embryos in culture were placed on the warm stage of a microscope and photos were taken every twenty seconds as they developed. All the events that took place over a period of 18-24 hours were observed subsequently and when the film was shown at normal projection speed it took only 3-4 min. Locations and movements of individual structures and cell groups were traced throughout the entire period of development (DeHaan, 1967). Although several cell types make up the embryonic chick heart _in vivo_, Polinger (1973a) found only two cell types in cultured chick heart cells. It has been demonstrated that contractile chick heart cells containing myofibrils are capable of division both _in vitro_ (Chacko, 1973; Weinstein and Hay, 1970; and Polinger, 1973b) and _in vivo_ (Polinger, 1973b). Kelly and Chacko (1976) observed a persistence of beating and maintenance of myofibrils with intact Z bands throughout division in cultured cardiac chick cells. Using high resolution phase contrast optics, Kasten (1972) has clearly shown that mitosis occurs in differentiated myocardial cells cultured from neonatal rats.

CHAPTER III

MATERIALS AND METHODS

All tissues used were from New Zealand White rabbits. Bred and virgin adults were obtained from Wilson's Rabbitry, Roopville, GA. The rabbits were sacrificed by cervical dislocation. Preimplanted (4-6 3/4 days) blastocysts and implanted embryos (7-10 days) were removed and placed in Petri dishes. Hearts and livers were excised from the adult and 10-day-old embryos, washed in ice-cold saline and stored at - 20^{o}C.

A. Isolation of Cardiac Myosin

Adult rabbit hearts were removed, weighed and chilled in ice-cold saline. The hearts were homogenized in 5 ml/heart of Weber solution (0.6 M KCL; 0.04 M $NaHCO_3$; 0.01 M Na_2CO_3 and 0.25 mM thioglycollic acid) in a blade homogenizer (14,000 rpm) for 1 min. The measured volume was adjusted to 8 ml/heart with Weber solution and addition of Triton-X 100 to a final concentration of 0.5%. The solution was then allowed to stand on ice for 15 min. The homogenate was centrifuged at 10,000 rpm (12,000 x g) for 10 min in a Sorvall centrifuge.
The supernatant was removed and centrifuged for 4 hr at 45,000 rpm (133,000 x g) in a Spinco centrifuge using Ti-50 rotor. After centrifugation the supernatant was poured into 10 volumes of ice-cold deionized water and the pH was adjusted to 6.8 with 1 N Na-acetate buffer (pH 4.5). The mixture was left overnight in the cold (4°C) to allow the precipitate to settle. The mixture was centrifuged at 10,000 rpm for 10 min. The resulting precipitate was dissolved in 6.5 ml of ATP solution

(0.3 M KCl; 0.01 M imidazole; 0.005 M $MgCl_2$; 0.005 M Na_2ATP; pH 6.8) per heart and centrifuged at 19,000 rpm (43,000 x g) in a Sorvall for 30 min; it was precipitated by the addition of 8 volumes of deionized water. The precipitate was allowed to stand for 1 hr on ice and centrifuged 10 min at 10,000 rpm. The pellet was then dissolved in 1.7 ml/heart of 0.5 N NaCl, 0.01 M imidazole buffer, pH 6.8. To remove any other aggregates and undissolved materials, two centrifugations were carried out, one for 10 min at 10,000 rpm and one for one hr at 100,000 x g. The resulting supernatant containing cardiac myosin was then frozen at -20^{o}C.

B. Isolation of Cytoplasmic Myosin

Adult rabbit livers were removed, washed with 30-50 ml of cold 0.027 M sodium citrate in calcium-free Locke's solution pH 7.2-7.4, pressed lightly within the folds of filter paper, then weighed and cut into several small pieces with a pair of scissors. A known wet weight of liver was transferred with 5 volumes of cold 0.25 M sucrose and homogenized. The suspension was filtered through cheese cloth to get rid of debris, and centrifuged at low speed (100-200 x g) for 2 min. The supernatant was then centrifuged at 68,000 x g. After removal of the supernatant, the pellet was resuspended in a known volume of homogenization buffer (0.15 M KCl, 0.006 M Tris-Cl, 2 mM sodium tetrathionate, 0.5 mM phenylmethylsulfonyl fluoride, pH 7.5) and frozen. The frozen liver suspension was thawed, suspended in 2 volumes of homogenization buffer, and homogenized with a Potter-Elveghom homogenizer, adjusted with homogenization buffer to 5 ml/ml of original packed tissue, and centrifuged at 68,000 x g for

1 hr. The supernatant was removed and the pellet resuspended in buffer (0.15 M KCl, 0.02 M Tris-Cl, pH 7.5). The resuspended pellet was adjusted to a final volume (4 ml per ml of suspension) with a concentrated extraction buffer (1 M KCl, 0.125 M Tris-Cl, 3.4 mM phenylmethylsulfonyl fluoride, pH 7.5). The mixture was stirred for 15 min and centrifuged at 68,000 x g for 1 hr. The supernatant was removed and the pH was adjusted to 6.5 with saturated maleic acid and stirred overnight in two volumes of cold distilled water. The mixture was then centrifuged for 30 min at 10,000 x g. The supernatant was discarded and the pellet resuspended in a small volume of 0.6 M KCl, 0.025 M Tris-Cl, 0.5 mM phenylmethylsulfonyl fluoride, pH 7.5. The resuspended pellet was dialyzed in dialysate buffer (0.6 M KCl, 0.025 M Tris-Cl) overnight and centrifuged at 95,000 x g for 30 min to obtain the soluble fraction (supernatant).

C. Measurement of Protein

Protein determination was done according to the methods of Lowry et al. (1951) and/or Murphy and Kies (1960). The latter method is based on peptide bond absorption which occurs in the 195-225 nm range. Absorbance was measured with a Cary 17 Spectrophotometer. The protein concentration was determined by the absorbance of proteins at 215 and 225 nm and was calculated according to the following equation:

$$\text{mg protein per ml} = A_{215} - A_{225} \times \text{Constant}$$

The absorption difference was used to minimize errors resulting from non-protein compounds in the solution. The constant for our protein was 0.154.

D. Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

1. Vertical Slab

Sodium dodecyl sulfate polyacrylamide slab gel electrophoresis (SDS-PASGE) was carried out using methods of Laemmli (1970). The stock solution by weight was 30% acrylamide and 0.5% N,N, bis-methylene acrylamide. The separation gel, using the slab gel technique, was composed of 7.5% acrylamide.

The 7.5% separating gel consisted of the following: acrylamide stock, 10.0 ml; Tris buffer (1.0 M, pH 8.8) 16.0 ml; SDS (20%) 0.4 ml; and deionized water, 11.6 ml. These solutions were mixed first, followed by the addition of 2.0 ml of ammonium persulfate and 20 µl of N,N,N',N' tetramethylethylenediamine (TEMED). Thirty-five millilters of this material were quickly poured into a slab gel chamber, overlayed with electrophoresis buffer, pH 8.2, and allowed to polymerize for two hr.

After the separating gel had polymerized, the electrophoresis buffer was removed and the gel was washed three times with distilled water. A 3% acrylamide stacking gel consisting of 2.0 ml of acrylamide stock, 1.69 ml Tris buffer (1.0 M, pH 6.8), 0.13 ml of SDS (20%); and 9 ml of deionized water was used. These components were mixed and 0.52 ml of ammonium persulfate and 10 µl of TEMED were added to the mixture. About 12 ml of the 3% stacking gel mixture was then poured on the separating gel. A 20-well comb was placed into the stacking gel and allowed to polymerize for 30-45 min.

Following polymerization, the comb was removed and the wells were made distinct by removing the excess solution (by use of several strips of filter paper) and placed in a vertical gel stand. The electrophoresis buffer contained 2 M Tris, 2 M gylcine (F.W. 75.07%), 20% SDS and distilled water up to 1000 ml. The pH was adjusted to 8.3, and buffer was added to the slab gel reservoirs. The samples were solubilized by boiling for 2-3 min in a solution containing 10% SDS, 15% glycerol, 2% mercaptoethanol and 0.004% bromophenol blue and added to the wells.

After the samples were added to the wells, the electrophoresis chamber was connected to a power supply with constant current and electrophoresed for about 15 hr at 15mA. Upon completion of electrophoresis, the stacking gel was discarded; the separating gel was then removed, fixed and stained according to the methods of Laemmli (1970).

2. Vertical Tube

For a typical run of 12 gels, 15 ml of gel buffer (8.82 g of $NaH_2PO4 \cdot H_2O$ (monobasic); 38.6 g of $Na_2HPO_4 \cdot H_2O$ (dibasic) and 1.0 g of sodium dodecyl sulfate in a final volume of 1 liter at a pH of 7) were mixed with 13.5 ml of 10% acrylamide solution. Freshly made ammonium persulfate solution (1.5 ml of 15 mg per ml) and 0.045 ml of N,N,N',N' tetramethylethylenediamine were added. After mixing, each tube was filled with 2 ml of the solution. Before the gel polymerized completely, a few drops of water were layered on top of the gel solution. After 10 min an interface indicated that the gel had solidified. Just before using, the water was gently removed with a Pasteur pipette, and the tubes were placed in the electrophoresis apparatus. The elecrophoresis chamber was filled with gel buffer diluted 1:1 with distilled water.

Gels were prepared using a 10% acrylamide solution, (22.2 g of acrylamide were mixed with 0.6 g of bis-methylene acrylamide and dissolved in 100 ml of distilled water). After mixing, the solution was filtered through Whatman #1 filter paper. The filtrate was stored in a dark bottle and kept refrigerated at 4°C.

For each gel, 3 µl of tracking dye (0.05% bromophenol blue in water), 1 drop of glycerol, 5 µl of 0.1% dithiothreitol (DDT), and 50 µl of the cardiac myosin or cytoplasmic myosin for electrophoretic analysis were added to small test tubes. After mixing, the individual samples were applied to the gel. Gel buffer diluted 1:1 with distilled water was carefully layered on top of each sample to fill the tubes.

Separation of protein was accomplished by discontinuous electrophoresis gels. Electrophoresis was performed at a constant current of eight (8) milliamps (ma) per gel, with the positive (+) electrode in the lower chamber. The single cell operation had a capacity of 60 volts, yielding a total voltage of 720.

After the run, the gels were allowed to stand 10 min. They were removed by inserting an 18" guage needle attached to a 50 ml syringe (filled with water). The needle was inserted through the bottom of the tube between the wall of the tube and the gel. Applying slight pressure, the gels were floated out of the electrophoretic tubes into individual staining tubes.

The gels were placed in small tubes filled with staining solution (1.25 g of Commassie Brilliant Blue dissolved in 454 ml of 50% methanol and 46 ml of glacial acetic acid). Insoluble materials were removed by filtration using Whatman #1 filter paper. The gels were stained at room temperature for 24 hr.

The gels were removed from the staining solutions, rinsed with distilled water, and placed in destaining solution composed of 75 ml of acetic acid and 50 ml of methanol in a final volume of one liter. The gels were destained for two days by changing the destaining solution routinely. After destaining, the gels were stored in 7.5% acetic acid solution.

E. Antibody Formation

Antibody formation, bleeding the animal by cardiac puncture and preparation and preservation of serum were done according to methods of Campbell et al. (1970) with modifications by Broadway (1978).

Injection of myosin: The hair was removed from the area of the animal to be injected by using electrical animal clippers. The animal was held against a horizontal surface, exposing the site to be injected, and the injection site was wiped with alcohol. The following sites were injected using 1", 26 guage needles.

1. Subcutaneous injections: The skin was raised with the thumb and index finger of the left hand. With the right hand, as much of the needle as possible was inserted underneath the skin, parallel to the underlying muscle. After an injection of cardiac myosin, the needle was quickly withdrawn.
2. Intracutaneous injections: The skin of the animal was stretched between the thumb and the index finger of the

left hand. Holding the syringe with the right hand at an angle of 30^{o} to the surface of the skin, the needle was inserted just below the outer skin layer.

3. Footpad injection: The guinea pig was inoculated intracutaneously through the foot pads. The needle was inserted through the center of the sole until resistance against the needle ceased. Cardiac myosin was injected and the needle removed. Care was exercised in order to insure that the needle did not lodge against the metatarsals.

Bleeding the Animal by Cardiac Puncture: Immunized male guinea pigs were bled by cardiac puncture. Between 2.5 ml to 5.0 ml of blood was taken during trial bleeding and 10 ml during major bleedings. The serum was decanted, centrifuged and stored at $-20^{o}C$ until used.

After the guinea pig was placed in a supine position and completely immobilized, the cardiac puncture procedures were as follows:

1. The guinea pig was shaved over the thorax and about 5 cm below the sternum, and the area wiped with ethyl alcohol.
2. After locating the xiphoid process and the last sternal rib to the left of the midline of the animal, the needle was inserted at a 30^{o} angle, just caudal to the point of junction of these two structures.
3. With the needle and syringe kept in this position, the needle was slowly advanced until the pulsation of the heart was felt. Blood (10 ml) was collected by slowly withdrawing the syringe plunger.
4. After the blood was collected, the needle was withdrawn

from the animal. The needle was removed from the syringe and with the flow of blood directed against the wall of the centrifuge tube, the syringe was emptied. Note: Lysis is reduced by avoiding foaming during delivery of the blood into the tube.

5. Separation of the serum was performed by Method D (Campbell et al.,1970). Freshly drawn blood was allowed to stand 1 to 2 hr at room temperature for clot formation. The clot was carefully separated from the wall of the tube with a steel spatula and stored in the refrigerator for 12 to 24 hr to allow clot retraction. The serum was poured into clean tubes and centrifuged twice at 100 x g for 30 min at about 4^{o}C to be sure to remove all erythrocytes and white cells. The serum was frozen at -20^{o}C for subsequent use.

F. Immunodiffusion

Immunodiffusion analyses were carried out according to the methods of Ouchterlony, (1967) as modified by Davidson, (1973). The agar gel consisted of a 1% solution of Agarose in phosphate buffered saline (PBS), pH 7.2, containing 0.1% sodium azide. These components were heated to facilitate solubilization of the agar. Ten milliliters of the molten agarose solution were dispensed into sterile 100 mm x 15 mm plastic Petric dishes, and allowed to solidify at room temperature. The plates were stored at 4°C in a moist chamber.

Before use, the plates were removed and allowed to warm to room temperature. Wells were cut in the agarose with a distance of 1 cm between the inner and outer wells. The agar plugs were then removed from the wells by aspiration and the wells were sealed. The wells were filled with the appropriate antigen or antiserum (10 µl), and refilled after the samples had diffused into the agarose. Following the addition of the reactants the plates were covered and incubated at room temperature in a humid chamber and examined daily over a period of five days for production of precipitin bands. The plates were then washed several times with distilled water at 4°C to remove the saline. Following the last distilled water wash, the plates were allowed to dry at room temperature, stained with Amido Black (Wieme, 1965), destained, and photographed with Kodak Pan-X black and white film.

G. Immunoelectrophoresis

Immunoelectrophoresis (Scheidegger, 1955) was performed by dissolving 1 g of Difco Noble Agar in a solution containing 99 ml of borate buffer (pH 8.5) and 1 ml of sodium azide. The buffer was prepared by adding 3.10 gm of boric acid; 4.80 gm of $NaB_4O_7 \cdot 10 H_2O$ and 2.40 g of sodium chloride to 1.5 liters of distilled water. The pH was then adjusted to 8.5 by the addition of 0.1 N HCl.

To prepare agar-coated slides, 2.5 ml of the dissolved Noble agar was applied to several 25 x 75 mm microscope slides with a 5 ml pipet. The agar was allowed to solidify at room temperature, and was then placed in a humidified chamber over night. An additional 20 ml of agar was pipetted over the slides and spread with the pipet tip to give a uniform layer. The agar was allowed to harden before cutting the appropriate wells with a Gelman gel punch.

The sera were placed in the proper wells using a 500 µl pipet, and the slides were placed across the bridges of a Gelman Deluxe Electrophoresis Chamber. The reservoirs were filled with borate buffer. Pieces of cotton gauze (soaked in buffer) were placed in the reservoirs with one end of the gauze making contact with the agar coated slides. The samples were electrophoresed for 120 min at 50 volts.

After the time required for electrophoresis had elapsed (i.e., 120 min) wells on both sides of the electrophoresed serum were made and filled with anti-serum. The slides were then incubated in a moist chamber for 24-72 hr at room temperature to allow for formation of precipitin bands. Once the precipitin bands were formed, the slides were washed and stained with 0.25% Commassie Brilliant Blue and photographed with Kodak Pan-X black and white film.

H. <u>Quantitative Precipitation</u>

Quantitative precipitation is the testing of a sample e.g. antibody (anti-cardiac myosin) and antigen (cardiac myosin) to determine the content of each substance. A constant amount (0.1 ml) of antigen and/or antibody is added to a series of tubes, followed by varying amounts of antigen and/or antibody from 10 µg to 200 µg. Saline (0.9%) was added to each tube to bring the volume up to 1 ml. The tubes were then incubated at 37°C for two hours and at 4°C overnight. Following the overnight incubation, the antigen-antibody pellet was washed 3 times with 0.9% saline. Each was followed by vortexing and centrifugation for fifteen min at 8,000 x g. After the last centrifugation the supernatant was withdrawn and the pellet was solubilized in 0.2 ml of 1N sodium hydroxide (NaOH) and 0.8 ml

0.9% saline to yield a final volume of one ml. The optical density (O.D.) at 280 nm was determined for the samples and a quantitative immunoprecipitation curve was constructed by plotting the optical density against the amount of cardiac myosin and/or anticardiac myosin added to each tube, to determine the equivalence point (that point where all the antigen and antibody are bound), and antigen excess and antibody excess regions.

I. Immunoprecipitation

Immunoprecipitation was carried out according to the methods of Goldman and Blobel (1981) as modified by Browne and Lowe (personal communication). To 1.5 ml Eppendorf tubes, 100 µl of cytoplasmic myosin (non-immune serum) or cardiac myosin (immune serum) was added along with 1.25 µl of 25% SDS and 10 µl of Aprotinin, a protease inhibitor which prevents degradation of the protein. The samples were boiled for 2 min, cooled and four volumes of a 1.25% Triton-X 100 buffer were added. The samples were vortexed thoroughly, 100 µl of the product from the above tubes were transferred into new tubes, followed by the addition of 10 µl of specific antiserum (anti-cardiac myosin). Once the antiserum was added, the product was vortexed well and 40 λ of Protein A-Sepharose per 10 λ of antiserum were added. Protein A-Sepharose is used for isolation of IgG antibodies and their fragments which contain the Fc regions. Vortexing and incubation at 37°C in a water bath followed by incubation on a rotating tube rotator for 2-3 hr, allows the mixture to form an antigen-antibody complex. The product after incubation was then centrifuged three times for 2 min at 12,000 x g. The supernatant was removed each time and 100 μl of 1% Triton-X 100 buffer were added. After the third centrifugation, 50 λ of 4% SDS were added, the samples were boiled 2-3 min, cooled, centrifuged at 12,500 x g for 2 min and prepared for loading onto a 1.5 mm thick 7.5 or 8.0% SDS-PAGE slab gel and electrophoresed at 20 mA for 16-20 hr.

J. Histology

Tissue from pregnant New Zealand White rabbits was removed and immediately placed in neutral buffered formalin fixative for 12 to 16 hr. The tissue was dehydrated through a graded series of ethyl alcohol solutions: 35%, 50%, 70%, 95% and 100%, for 30 min each. Then the tissue was cleared in a 1:1 mixture of toluene and paraffin (2 changes) for 30 min each. The tissue was then placed in melted paraffin (2 changes) for 3 min each, infiltrated for 4 hr in a vacuum infiltrator, and then embedded in paraplast. After embedding the tissue in paraplast and allowing it to harden overnight in plastic peel away boats, sections were cut at 5 to 15 μm on an AO Spencer "820" rotary microtome. The sections were then floated onto labeled slides, dried and stained with Harris' Hematoxylin and Eosin.

K. *In Vitro* Studies

Preimplanted blastocysts (5 3/4-6 3/4 day p.c.) were removed from the uteri of pregnant New Zealand White rabbits. At this stage the blastocysts have two morphologically distinct areas, the trophoblast and the inner cell mass. The blastocysts were cultured in one of three ways:

1. Whole blastocysts with zona pellucida intact
2. Blastocysts were fragmented using fine tipped forceps and

3. The zona pellucida was removed and the inner cell mass and trophoblast were separated (Coleman, 1977) and placed in separate culture dishes.

All samples were placed in Falcon plastic Petri dishes (35x10 mm) containing 3 ml of Waymouth's MB 752/1 culture medium, supplemented with 10% fetal calf serum, 5% human serum and 100 units/ml penicillin-streptomycin (GIBCO Laboratories). The Petri dishes were incubated in a National Model 3221 water-jacketed CO_2 incubator at 36.5°C in an atmosphere of 5% CO_2-95% air. The cultures were observed using a Leitz inverted phase contrast microscope and photographed with a Leitz Leicina Special camera using Kodak Ektachrome 200 color film.

L. Immunofluorescence

1. Microscope Slide Method: Normal embryonic tissues (5 3/4, 6, 6 3/4-day-old blastocysts and 10-day fetal heart) were obtained from New Zealand White rabbits. The tissue was either quickly cooled in liquid nitrogen or placed into an optical temperature cutting (O.T.C.) compound and placed in the cryostat at -20°C. Sections were made from the frozen samples.

Sections were placed on slides that had been cleaned in 70% ethanol and sprayed with fluorocarbon. Thumb tacks were placed on the slides before spraying with fluorocarbon. After the slides dried, the thumb tacks were removed leaving clear, round circles where the tissue was later placed.

Indirect Staining Method: Tissue was sectioned at 1 1/2 µm to 2 µm and placed on coated slides. The slides were then placed in a slide holder and immersed in cold phosphate buffered saline (PBS) at pH 7.2 and refrigerated for 1 hour. After the antisera were added, using a 10 µl pipet the slides were incubated for 1/2 hour in a moist chamber at 4°C. Using a Pasteur pipette the antisera were carefully rinsed off with PBS and washed three times for 10 minute each in a slide chamber at room temperature. The slides were then blotted dry, taking care not to let the tissue dry out. A 1:8 dilution of the conjugated fluorescein isothiocyanate (FITC) anti-guinea pig IgG produced in rabbit (Miles Laboratory) was added and incubated for 30 min in a moist chamber at 4°C. The washing procedure using PBS was repeated in order to remove the FITC. The slides were then mounted in PBS/glycerol (in a ratio 1:2). The slides were then ready for observation or for storage in a light-tight box for a period not to exceed two months. The slides were photographed using a Leitz fluorescent microscope and a Leitz Orthomat camera using Kodak Ektachrome 400 color film.

2. Microscope Cover Slip Method: The cover slip method was carried out according to the method of Kaufman (personal communication). The embryonic disc was removed from the trophoblast and grown on sterilized 11 x 22 mm glass cover slips in 35 x 10 mm Petri dishes containing Waymouth MB 752/1 culture medium supplemented with 10% fetal calf serum, 100 units/ml penicillin-streptomycin and 5% human serum. The cover slip was removed from the media, rinsed in PBS pH at 7.4 and fixed for 20 min in 3% formaldehyde. After fixation of the cells, the cover slip was removed from the fixative, rinsed again in PBS and drained by blotting the edge of the upright cover slip. The cover slip with the embryonic disc attached was then placed

into cold acetone (-10° to -20°C) for seven min. The acetone was removed using PBS at room temperature and drained as above. Fifty µl of anti-cardiac myosin serum were placed in a 35 mm Petri dish and the inverted cover slip (with the cells in contact with antibody) was incubated at 37°C for 45 min. The cover slip was then removed from the Petri dish by adding a gentle stream of PBS to the dish floating the cover slip off the surface; the cover slip was rinsed well in PBS, drained and blotted. The cover slip, containing cells incubated with the anti-cardiac myosin serum, was inverted into a Petri dish containing 25 µl of a 1:6 dilution of fluorescent tag (anti-guinea pig IgG fluorescence tag for antibody produced in rabbit), in PBS and incubated at 37°C for 30 min. The cover slip was removed from the Petri dish and rinsed with PBS. Excess liquid was blotted and the cover slip (cell side down) was placed over a small drop of 1 part PBS plus 9 parts glycerol, pH 8.5, on a clean microscope slide. The samples were observed and photographed using a Leitz fluorescent microscope and a Leitz Orthomat camera using Kodak Ektachrome 400 color film. The samples are stable for two days if stored in a black box in the refrigerator at 4°C.

CHAPTER IV

EXPERIMENTAL RESULTS

A. Molecular Weight Determination of Cardiac and Cytoplasmic Myosin from Adult Rabbit Tissues

Polyacrylamide gel has advantages over other support media for electrophoresis because it is transparent, free from charged or reactive groups, has good mechanical properties and is easily prepared.

A control gel of canine skeletal muscle extraction is shown in Fig 1. Figures 2 and 3 show the electrophoretic separation of cardiac and cytoplasmic myosin from adult rabbit heart and liver respectively. Figures 4, 5 and 6 illustrate the electrophoretic profiles of skeletal, cardiac and cytoplasmic myosins. Table 1 is a comparison of various myosins whose heavy and light chains coincide with our data. Our results indicate that rabbit cardiac myosin has two heavy chains approximately 200,000 D each, a C-protein (function unknown) 40, 000 D, two light chains (LC), LC-1 25,000 D and LC-2 17,500 D; and cytoplasmic myosin has two heavy chains (HC) HC-1 150,000 D and HC-2 111,000 D, and two light chains (LC) LC-1 28,000 D and LC-2 19,000 D.

B. Immunodiffusion

Figure 7 shows identity using a known antigen and antibody. Examination of Fig. 8 shows identity between adult anti-cardiac myosin and adult rabbit cardiac myosin. Embryonic cardiac antibodies showed identity with embryonic cardiac antigens (Fig. 9).

Immunodiffusion provides a simple means for evaluating, to a limited extent, the basis for cross-reactions observed in liquid

Fig 1: Sodium dodecyl sulfate polyacrylamide gel electrophoresis of canine skeletal muscle protein. (A) Heavy chains, 200, 000 D each, migrating similarly; (B) C-protein, 84,500 D; (C) Actin, 42,000 D; (D) Tropomyosin, 35,000 D; and three light chains (E) LC-1 25,000 D; (F) LC-2 18,000 D and (G) LC-3 16,000 D.

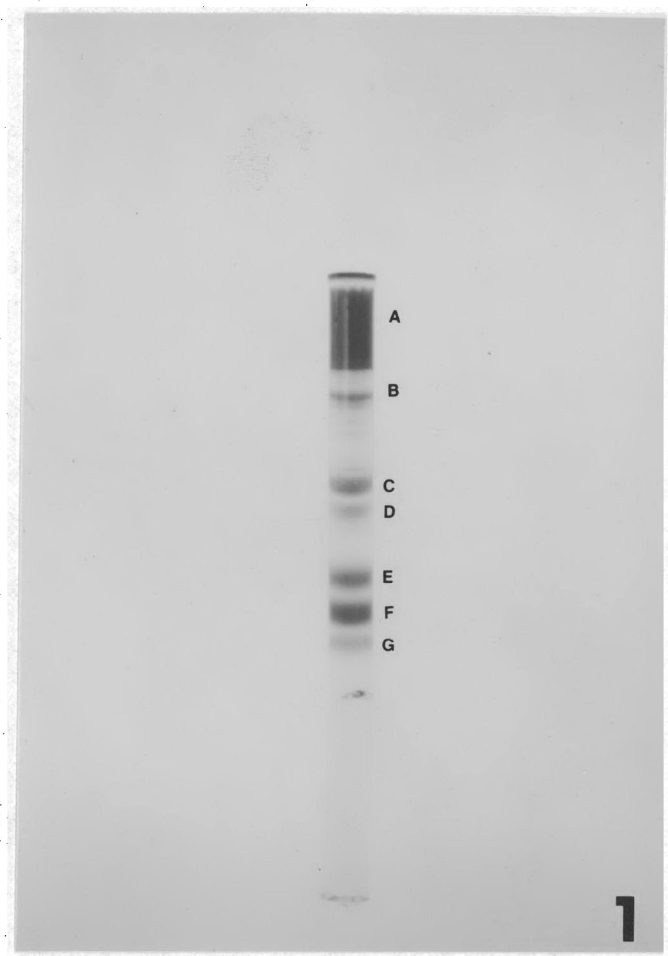

A
B
C
D
E
F
G
1

Fig 2: Sodium dodecyl sulfate polyacrylamide gel electrophoresis of adult cardiac myosin. (A) Two heavy chains 200,000 D each migrating similarly (B) C-protein 40,000 D , and two light chains (C) LC-1 (25,000 D) and (D) LC-2 (17,500 D).

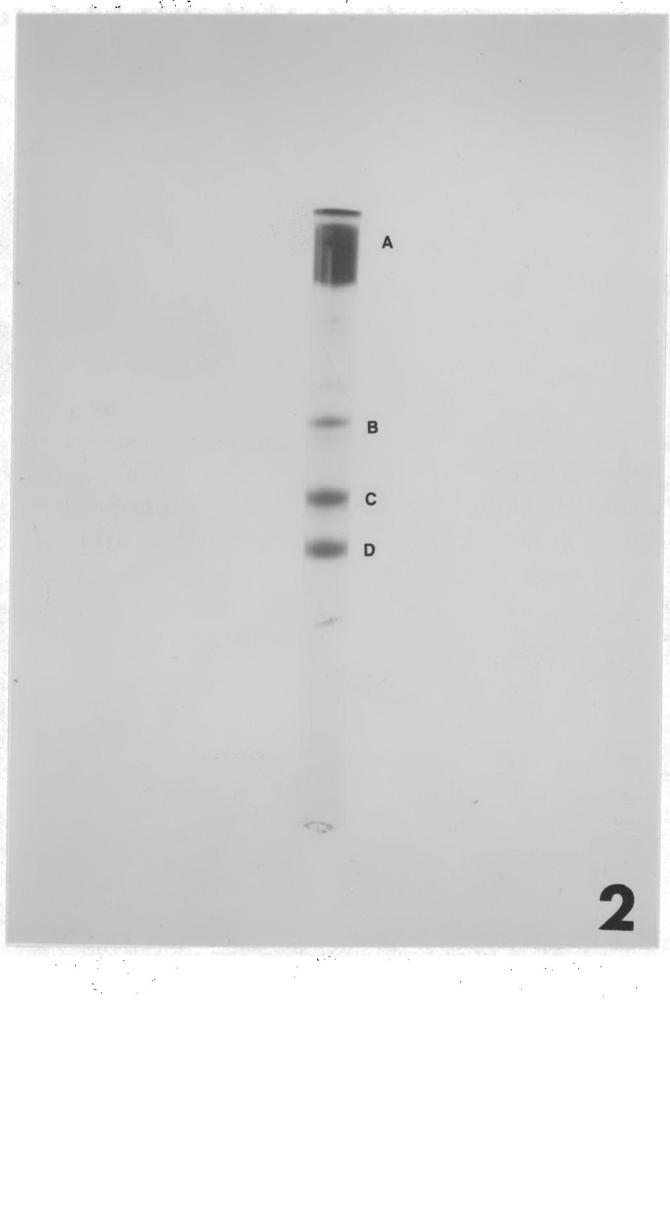

A
B
C
D
2

Fig 3: Sodium dodecyl sulfate polyacrylamide gel electrophoresis of rabbit liver cytoplasmic myosin. (A) Heavy chains HC-1 (150,000 D) and (B) HC-2 (111, 000 D) and two light chains (C) LC-1 (28,000 D) and (D) LC-2 (19,000 D). Note in non-muscle myosin the separation of two heavy chains A and B. This is not commonly seen in cardiac and skeletal muscle myosin.

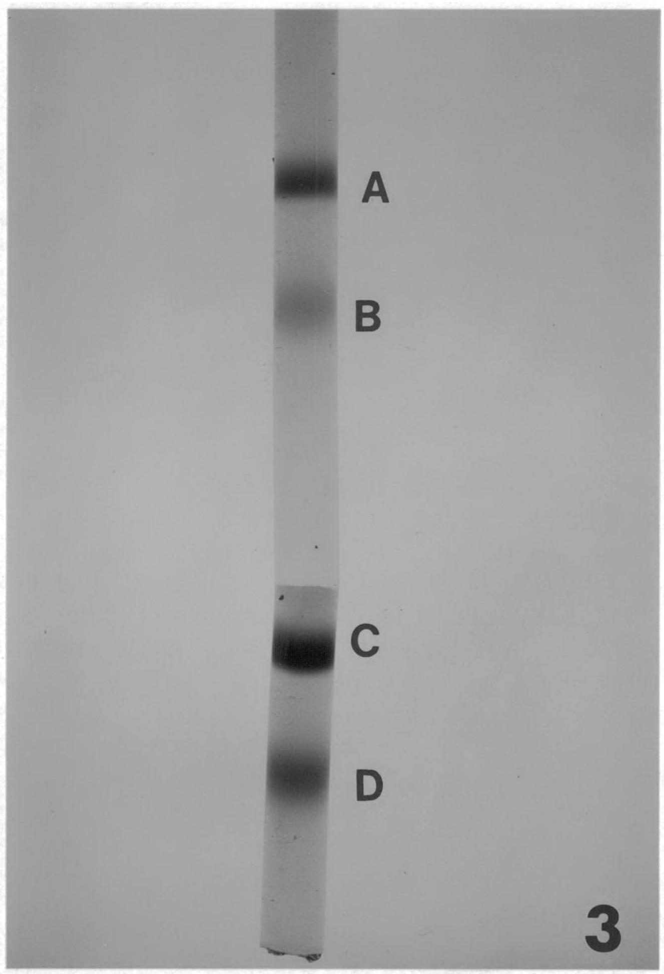

A
B
C
D
3

Fig 4: An electrophoretic profile of canine skeletal myosin. Heavy chain, 200,000 D; C-protein, 84,500 D; Actin, 42,000 D; Tropomyosin, 35,000 D; Light chain 1, 25,000 D; Light chain 2, 18,000 D and Light chain 3, 16,000 D.

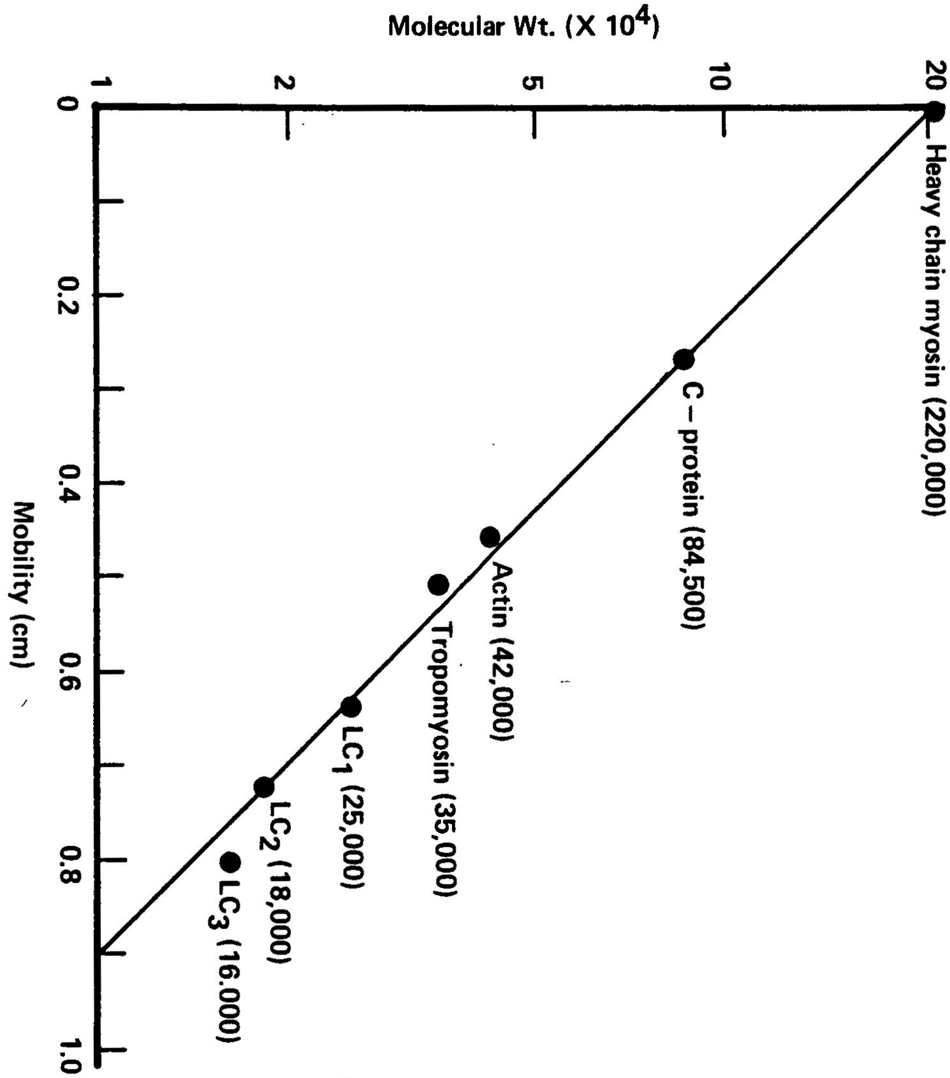

Molecular Wt. (X 10^4)
20
10
5
2
1
0
0.2
0.4
0.6
0.8
1.0
Mobility (cm)
Heavy chain myosin (220,000)
C – protein (84,500)
Actin (42,000)
Tropomyosin (35,000)
LC$_1$ (25,000)
LC$_2$ (18,000)
LC$_3$ (16.000)

Fig. 5: An electrophoretic profile of adult rabbit cardiac myosin with heavy chains (200,000 D), C-protein 40,000 D and two light chains 1 (25,000 D) and 2 (17,500 D).

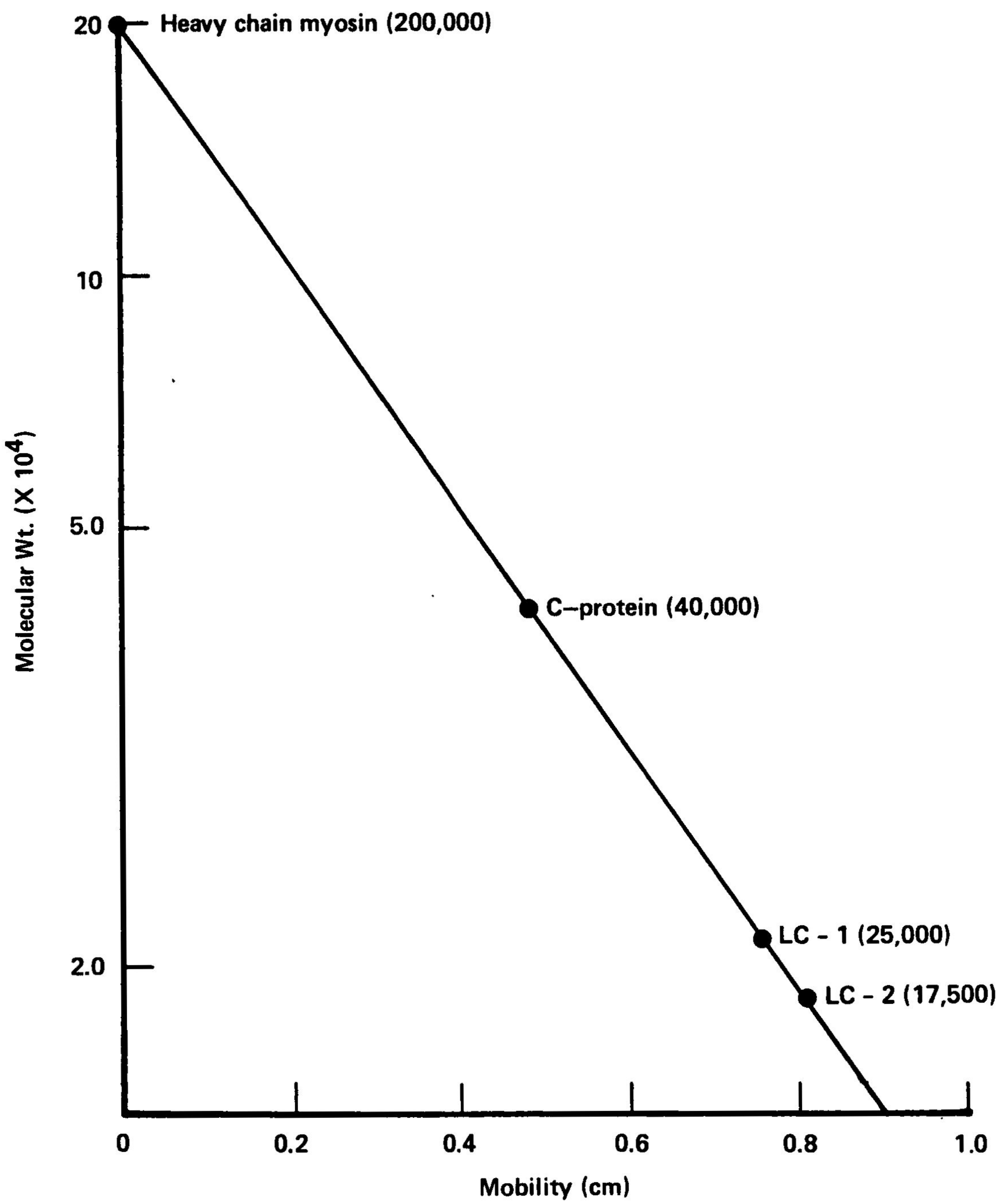

Heavy chain myosin (200,000)
C–protein (40,000)
LC - 1 (25,000)
LC - 2 (17,500)
20
10
5.0
2.0
Molecular Wt. (X 10⁴)
0
0.2
0.4
0.6
0.8
1.0
Mobility (cm)

Fig. 6: An electrophoretic profile of adult rabbit liver cytoplasmic myosin. Heavy chains 1 (150,000 D) and 2 (111,000 D) and two light chains 1 (28,000 D) and 2 (19,000 D).

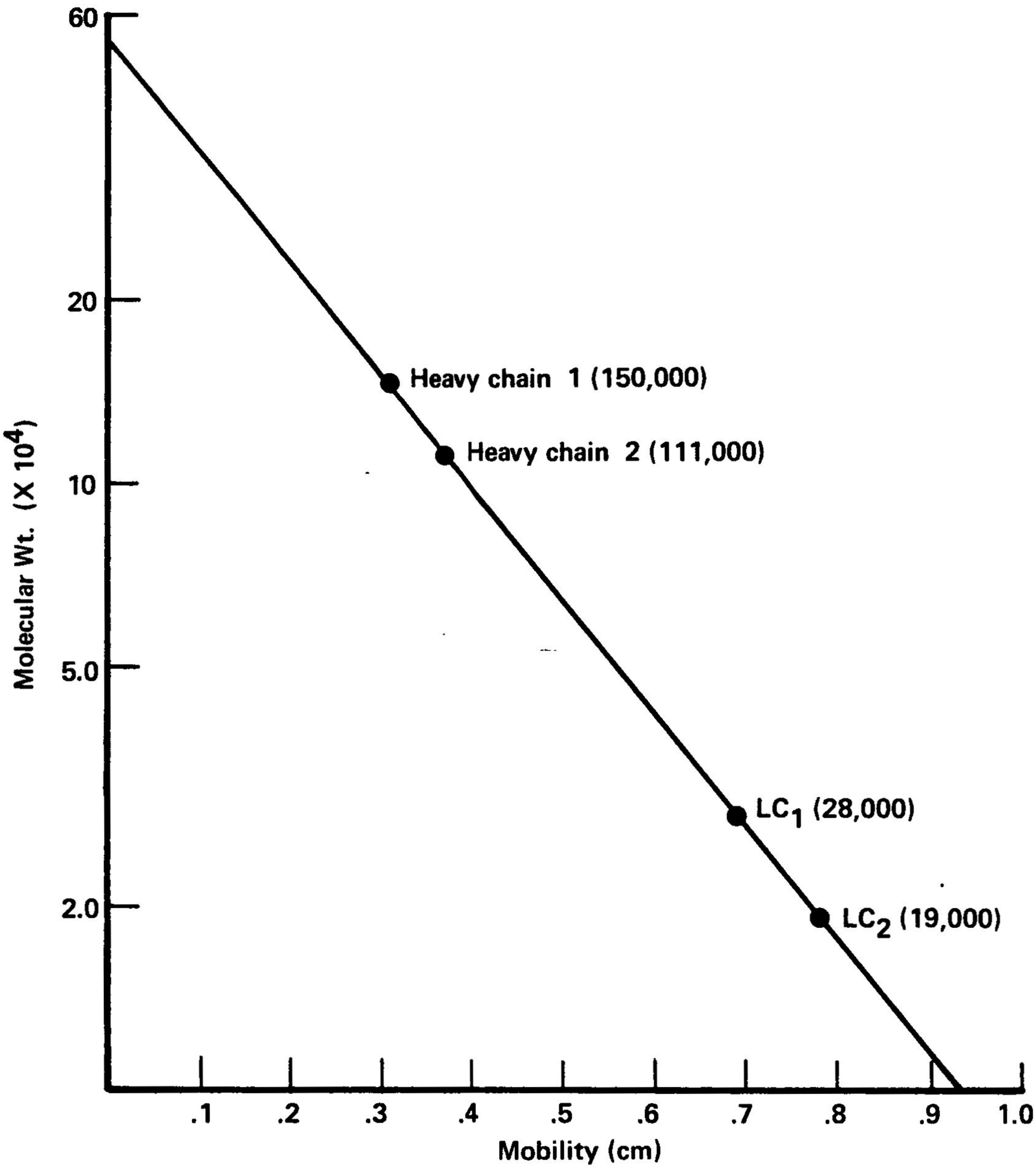

60
20
10
5.0
2.0
Molecular Wt. (X 10^4)
Heavy chain 1 (150,000)
Heavy chain 2 (111,000)
LC_1 (28,000)
LC_2 (19,000)
.1
.2
.3
.4
.5
.6
.7
.8
.9
1.0
Mobility (cm)

Table I: A comparison of several types of myosin.
Sodium dodecyl sulfate polyacrylamide gel electrophoresis of several types of myosin illustrating their native molecular weights and subunits. Information taken from Pollard and Weihing, 1974 (*) and our data (**).

TABLE 1. A comparison of several types of myosin.

TYPES OF MYOSIN	NATIVE MOLECULAR WEIGHT (DALTONS)	SUBUNITS (# x DALTONS)
1. Striated muscle		
rabbit skeletal*	420,000	2 x 200,000 1 x 27,000 1 x 16,000
rabbit cardiac**	442,000	2 x 200,000 1 x 25,000 1 x 17,500
2. Smooth Muscle		
human uterus*	?	? x 170,000 ? x 20,000
3. Vertebrate cytoplasmic		
guinea pig granulocyte*	?	? x 200,000 ? x 20,000
rabbit hepatocyte**	309,000	1 x 150,000 1 x 111,000 1 x 28,000 1 x 19,000
human platelet*	540,000	2 x 200,000 2 x 19,000 2 x 16,000
mouse fibroblast*	?	? x 200,000 ? x 20,000
rat brain*	?	? x 240,000
4. Physarium*	460,000	2 x 240,000 ? x 12,000
5. Acanthamobea*	180,000	1 x 140,000 1 x 16,000

* Data taken from Pollard, T. and Weihing, R., 1974
** Our data.

Fig. 7: Immunodiffusion analysis of anti-rabbit serum antibody produced in guinea pig (center well) against normal rabbit serum antigen.

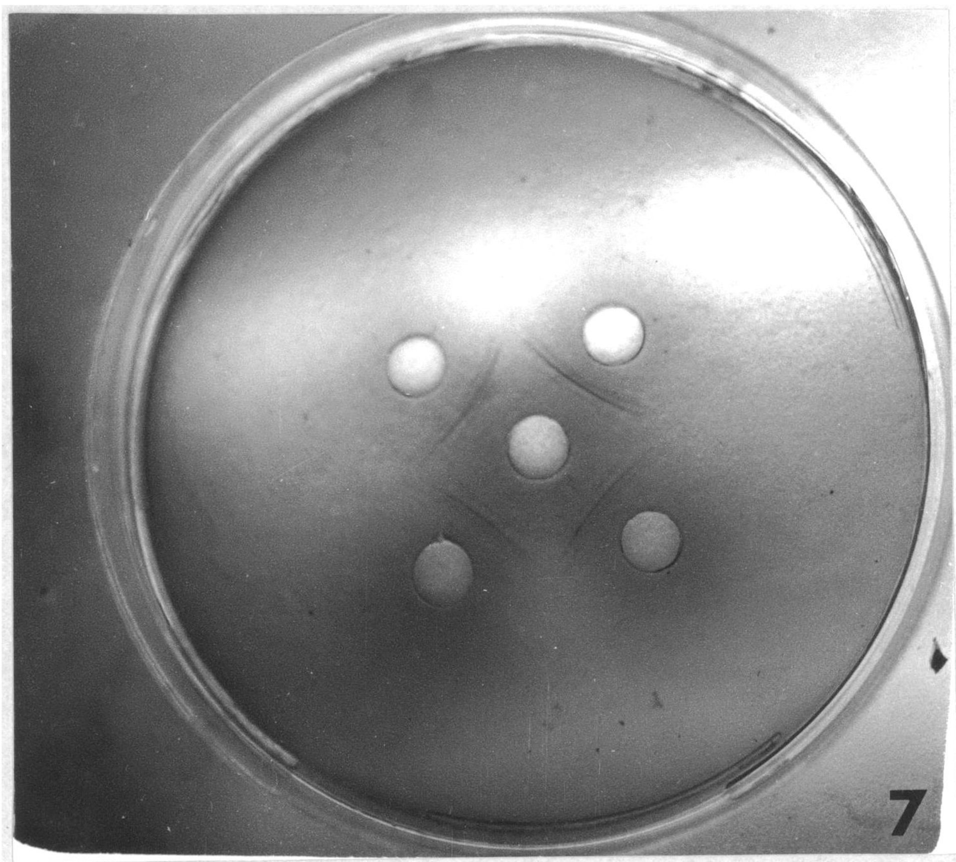
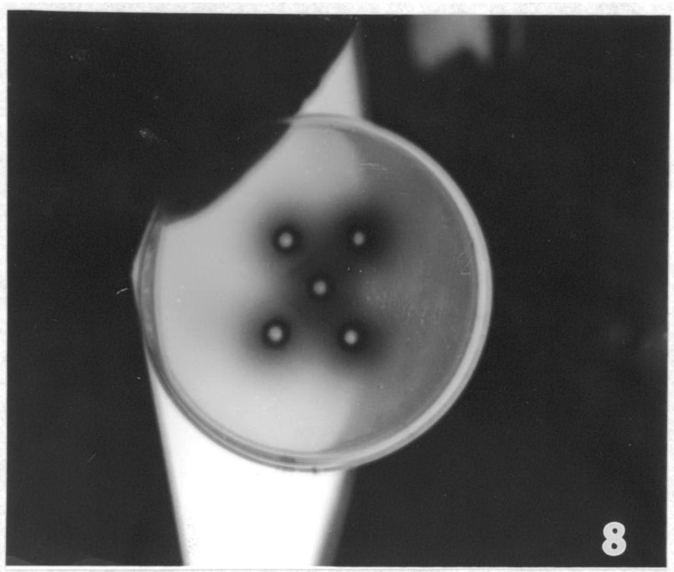
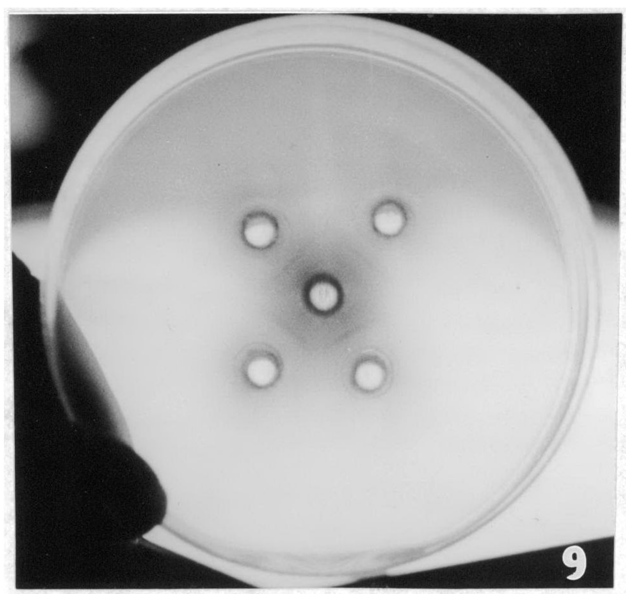

7

Fig 8: Immunodiffusion analysis of anti-cardiac adult rabbit myosin antibody. Anti-cardiac adult rabbit myosin antibody produced in guinea pig (center well) and adult rabbit cardiac myosin antigen in outer wells.

Fig 9: Immunodiffusion analysis of anti-embryonic cardiac rabbit antibodies. Anti-cardiac embryonic rabbit antibodies produced in guinea pig (center well) and rabbit embryonic cardiac antigens in outer wells.



8



9

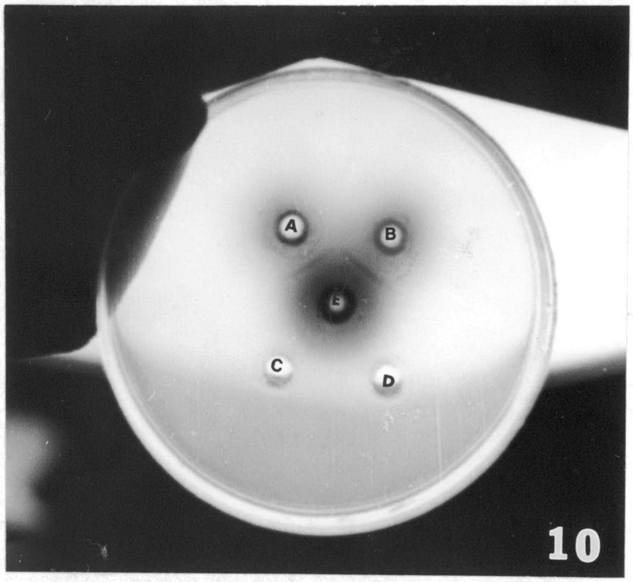
media. In the event that neither of the antigens are homologous with respect to the anti-serum, no cross-reactivity is observed. Figures 10 through 16 show multiple antigens and one antibody. When anti-rabbit adult cardiac myosin was allowed to react with cytoplasmic and cardiac myosins (adult and embryonic), visible precipitin bands were seen only between embryonic and adult cardiac myosins (Fig 10), therefore it can be concluded that there is no cross-reactivity between cardiac and cytoplasmic myosin. Using anti-cytoplasmic myosin antibodies and both cardiac myosin and cytoplasmic myosin as antigens, no cross reactivity is obseved between cardiac and cytoplasmic myosin (Fig 11). Figures 12 and 13 reveal that when embryonic antigens (cardiac and cytoplasmic myosins) were reacted with rabbit embryonic cardiac and cytoplasmic antibodies, respectively, identity again was visible only with its counterpart. When examining our anti-embryonic antibodies with adult and embryonic antigens (Figs 12 and 13), respectively, visible precipitin lines were seen. These results are further verified in Figs 14 and 15. Techniques for studying the sensitivity of antigen and antibody reactions in agar, allow us to conclude that cardiac myosin isolated in our studies does not cross react with cytoplasmic myosin extracted from the same species (Figs 16A and 16B).

C. Immunoelectrophoresis

Figure 17 shows identity by immunoelectrophoresis using a known antigen and antibody. Figure 18A shows with a concentration of 1:1 (adult anti-cardiac myosin against adult cardiac myosin), one gets a distinct precipitin antigen-antibody arc. Similar results were

Fig 10: Immunodiffusion analysis of anti-rabbit adult cardiac myosin antibody (E), raised against rabbit adult cardiac myosin antigen (A), rabbit embryonic cardiac antigens (B), adult rabbit cytoplasmic myosin antigen (C), and rabbit embryonic cytoplasmic antigens (D).

Fig 11: Immunodiffusion analysis of anti-rabbit cytoplasmic myosin antibody (E), raised against rabbit adult cardiac myosin antigen (A), rabbit embryonic cardiac antigens (B), rabbit embryonic cytoplasmic antigens (C) and rabbit adult cytoplasmic myosin antigen (D).


A
B
E
C
D
10

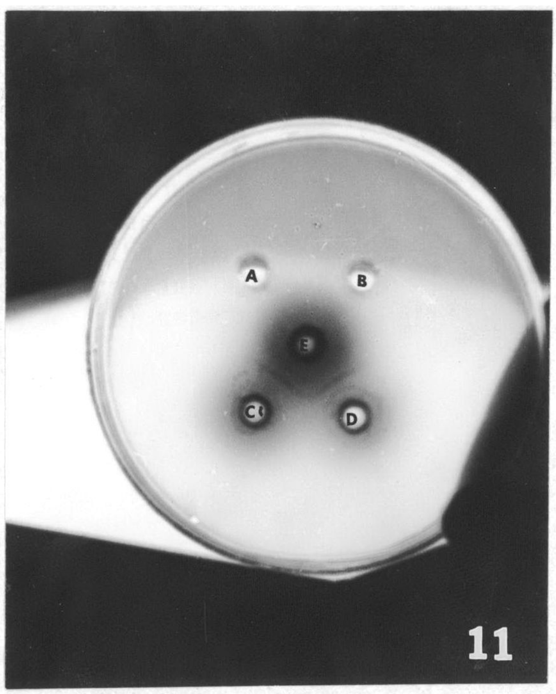
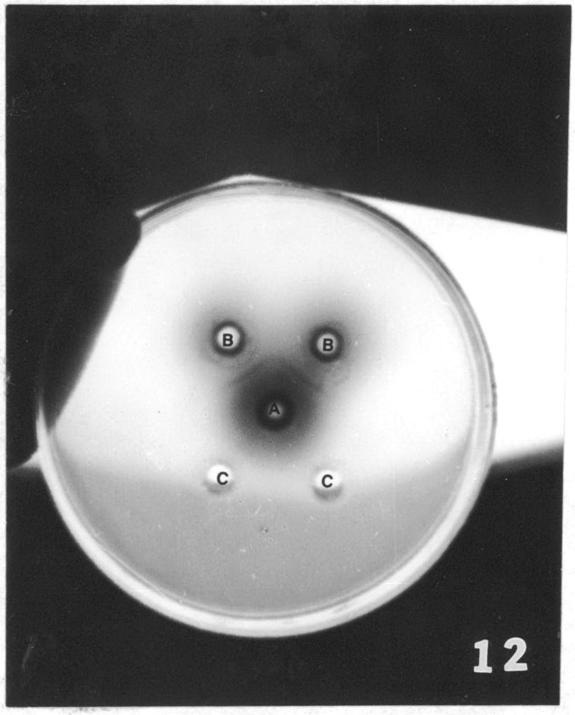
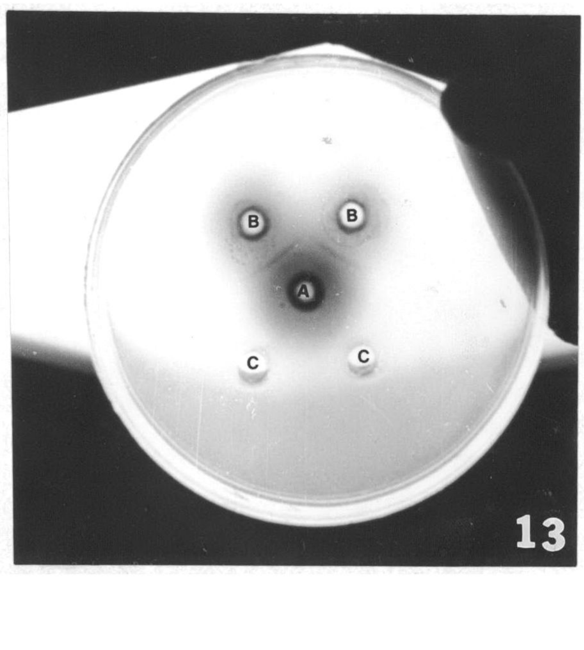

A
B
E
C
D
11

Fig 12: Immunodiffusion analysis of anti-rabbit embryonic cardiac antibodies (A) against rabbit embryonic cardiac antigens (B) and rabbit embryonic liver antigens (C).

Fig 13: Immunodiffusion analysis of anti-rabbit embryonic liver antibodies (A) against rabbit embryonic liver antigens (B) and rabbit embryonic cardiac antigens (C).



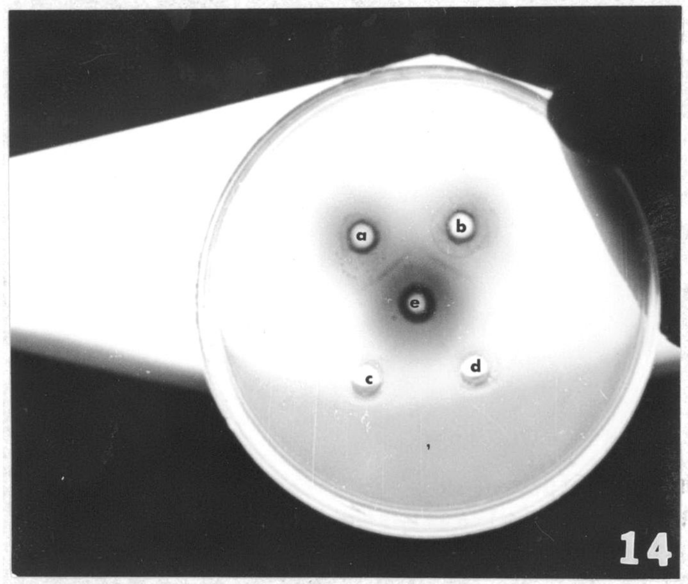
12



13

Fig 14: Immunodiffusion analysis of anti-rabbit embryonic cardiac antibodies (e) against adult rabbit cardiac myosin antigen (a), embryonic rabbit cardiac antigens (b), embryonic rabbit liver antigens (c) and adult rabbit liver cytoplasmic myosin antigen (d).

Fig 15: Immunodiffusion analysis of anti-rabbit embryonic liver antibodies (e) against adult rabbit cardiac myosin antigen (a), rabbit embryonic cardiac antigens (b), adult rabbit liver cytoplasmic myosin antigen (c) and rabbit embryonic liver antigens (d).


a
b
e
c
d
14

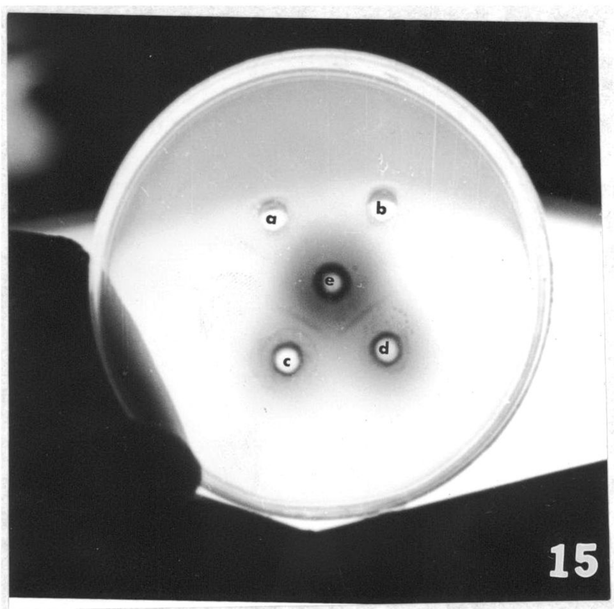
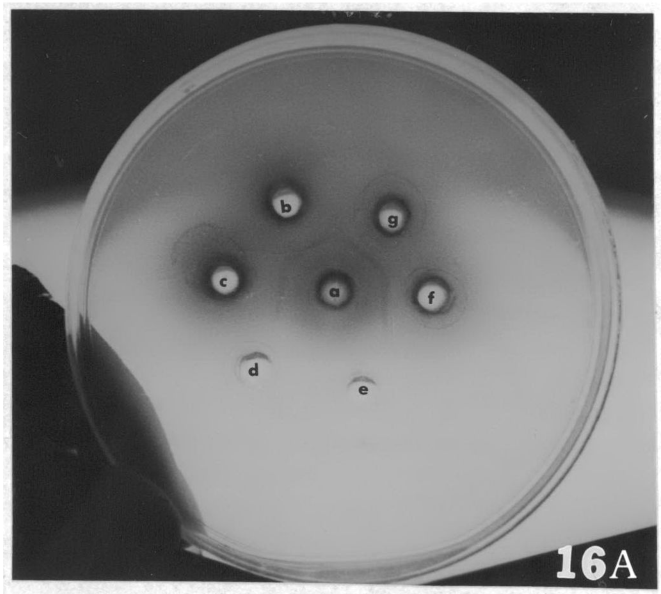

a
b
e
c
d
15

Fig 16: Immunodiffusion analysis of anti-rabbit adult cardiac myosin antibody and anti-rabbit adult cytoplasmic myosin antibody.

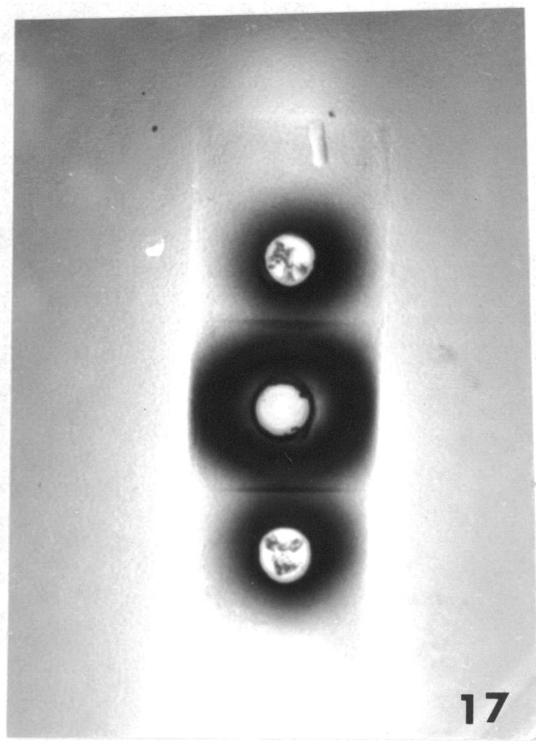
Panel A. An immunodiffusion analysis of anti-rabbit adult cardiac myosin antibody (A) against adult rabbit cardiac myosin antigen (B), embryonic rabbit cardiac myosin antigen (C), adult rabbit cytoplasmic myosin antigen (D), rabbit embryonic liver antigens (E), rabbit adult cardiac myosin antigen (F) and rabbit embryonic cardiac antigens (G).

Panel B. An immunodiffusion analysis of anti-rabbit adult cytoplasmic myosin antibody (A) against adult rabbit cardiac myosin antigen (B), rabbit cytoplasmic myosin antigen (C), embryonic rabbit liver antigens (D), adult rabbit cytoplasmic myosin antigen (E), embryonic rabbit liver antigens (F) and embryonic rabbit cardiac antigens (G).


b
g
c
a
f
d
e
16A


b
g
c
a
f
d
e
16B

Fig 17: Verification of specificity by immunoelectrophoresis using known anti-rabbit serum antibody (center well) against normal rabbit serum antigen (outer wells).


17

obtained with embryonic cardiac antigens against embryonic anti-cardiac antibodies (Fig 18B), and adult anti-cardiac antigen and embryonic cardiac antigens (Fig 18C), suggesting that anti-cardiac myosin is monospecific for cardiac myosin.

Since cytoplasmic myosin may be detected early in development; the reaction between antisera made to cardiac myosin and cytoplasmic myosin was performed. The following figures display composite immunoelectrophoretic patterns illustrating the relative position of proteins in agarose gels. Due to the variability in the positions of precipitin arcs, as well as their varying visibility under different experimental conditions, only certain major archs are identifiable. Figures 19A and 19B show identity between anti-cytoplasmic myosin and adult cytoplasmic myosin as well as between anti-liver cytoplasmic myosin and embryonic liver antigens. Reactions between anti-cytoplasmic myosin antibody and rabbit embryonic cardiac antigens, rabbit anticytoplasmic myosin and rabbit cardiac myosin, and anti-cardiac myosin and adult cytoplasmic myosin revealed no precipitin bands, indicating no cross reactivity (data not shown).

D. Quantitative Precipitation

Optimal concentration of antigen and antibody lead to extensive cross-linking of the molecules. The ultimate result is the formation of a three-dimensional latticework of antigen and antibody which precipitates out of solution. The proportion of antigen - to - antibody molecules that is present in the mixture determines the extent of cross-linking, and consequently the precipitation, that ultimately occurs. The precipitation curve is divided into three

Fig 18: Verification of Anti-Cardiac Myosin Antibody Specificity by Immunoelectrophoresis.

Panel A. Adult anti-cardiac myosin antibody (center well) against adult cardiac myosin antigen (outer wells).

Panel B. Embryonic anti-cardiac antibodies (center well) against embryonic cardiac antigens (outer wells).

Panel C. Adult anti-cardiac myosin antibody (center well) against embryonic cardiac antigens (outer wells).

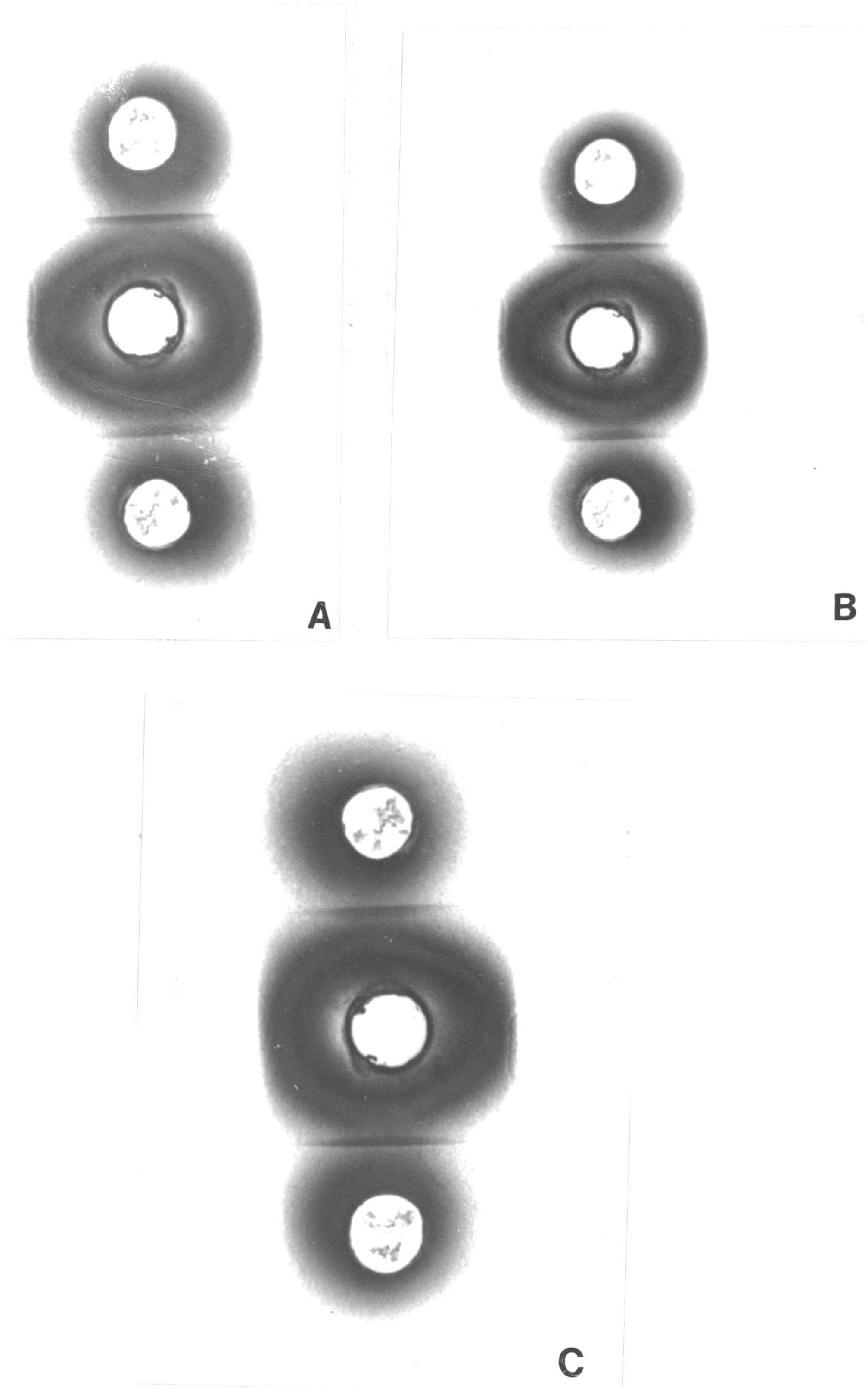
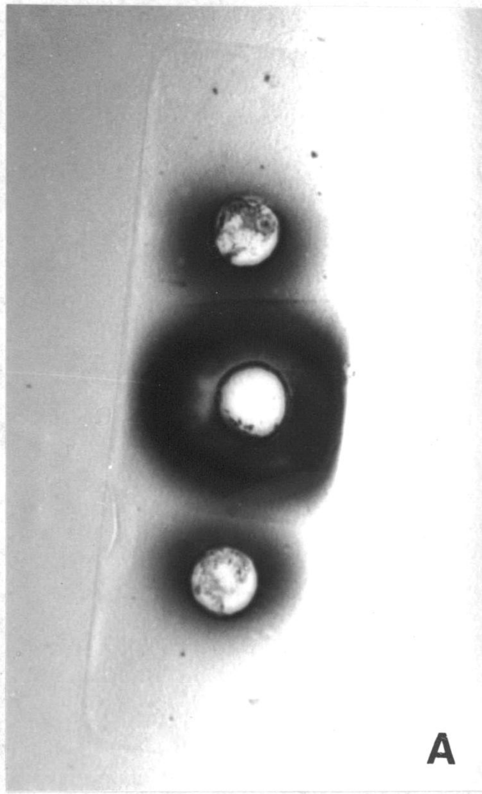
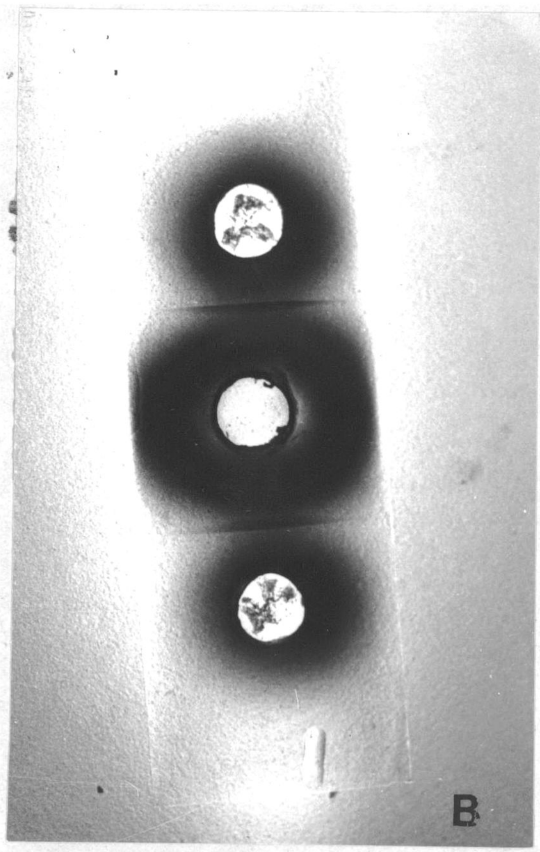

A
B
C

Fig 19: Verification of Anti-Cytoplasmic Myosin Antibody Specificity by Immunoelectrophoresis.

Panel A. Adult rabbit liver anti-cytoplasmic myosin antibody (center well) against adult liver cytoplasmic myosin antigen (outer wells).

Panel B. Embryonic rabbit anti-liver antibodies (center well) against embryonic liver antigens (outer wells).


A


B

regions: 1) antibody excess, 2) zone of equivalence and 3) antigen excess. When an optimal proportion of antigen to antibody is present, cross-linkage is extensive and large insoluble complexes are formed, which are seen as a visible precipitate. Soluble complexes are small antigen-antibody aggregates which are formed when all of the antigenbinding sites of antibody are bound by an individual antigen molecule (as in antigen excess) or, when all free antigenic determinants are occupied by an individual antibody molecule (as occurs in antibody excess).

In Fig 20, using constant concentrations of antigen (cardiac myosin) and varying concentrations of antibody (anti-cardiac myosin), it is seen that the optimal concentration of antibody to antigen is 90 μg/ml.

E. Histology

Figure 21 is a section of the whole 6 3/4-day blastocyst, showing the zona pellucida and embryo proper. Figure 22 is a section through the embryonic area of the 6 3/4-day blastocyst. Note the zona pellucida and the inner cell mass. Using Harris' Hematoxylin (Fig 23) the cytoplasm of the cells of the inner cell mass of the *in vitro* cultured blastocysts stained acidophilic and the nuclei stained basophilic. Figure 24 is a section through the 7 3/4-day implanted collapsed blastocyst within the intact uterus. Due to the rapid changes which take place during implantation, it becomes difficult for one to trace blastocyst development up to the point of formation of the heart. The cells that make up the inner cell mass of both the *in vivo* embryo and the *in vitro* cultured blastocyst, undergo rapid

Fig 20: Quantitative Immunoprecipitation using Cardiac Myosin Antigen and Anti-cardiac Myosin Antibody.

Increasing quantities of anti-serum (10, 40, 70, 100, 130, 160 and 200 ug) were added to separate sets of tubes. All volumes were adjusted to 1 ml with 0.9% saline. Cardiac myosin (0.1 ml) was added to each tube and the precipitate allowed to form at 37^{o}C for 2 hr and overnight at 4^{o}C. The precipitate was determined by plotting optimal density at 280 nm against the amount of anti-serum (μg protein/tube) added.

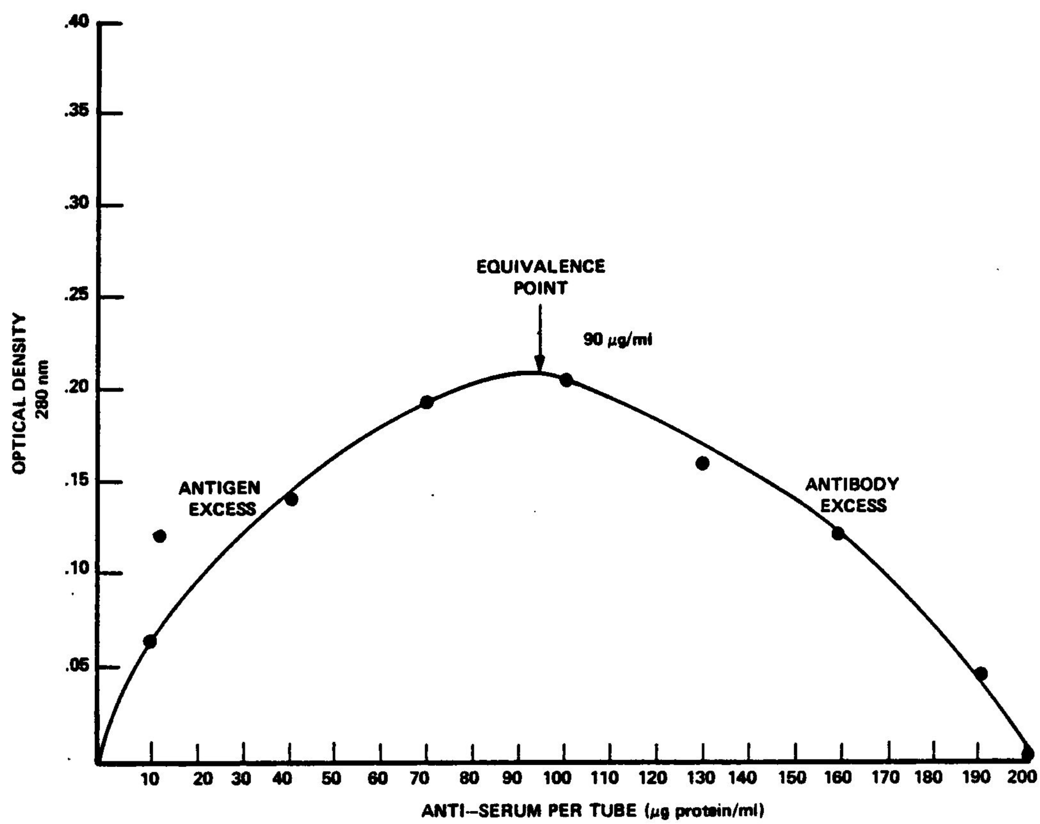

.40
.35
.30
.25
.20
.15
.10
.05
OPTICAL DENSITY
280 nm
EQUIVALENCE
POINT
90 μg/ml
ANTIGEN
EXCESS
ANTIBODY
EXCESS
10 20 30 40 50 60 70 80 90 100 110 120 130 140 150 160 170 180 190 200
ANTI–SERUM PER TUBE (μg protein/ml)

Fig 21: A section of the whole 6 3/4-day-old blastocyst with the zona pellucida (ZP) and the embryo proper (EP). Note, the trophoblast is not distinguishable from the inner cell mass. 25X

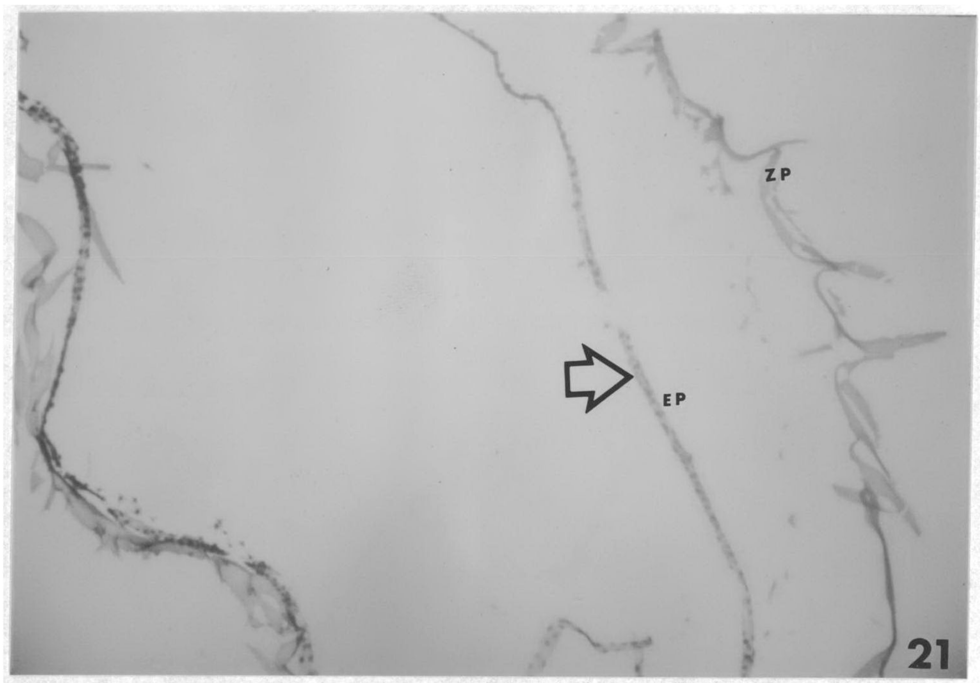

ZP
EP
21

Fig 22: A section through the embryonic area of the 6 3/4-day-old rabbit blastocyst. Note the zona pellucida (ZP) and the inner cell mass (ICM). 64X

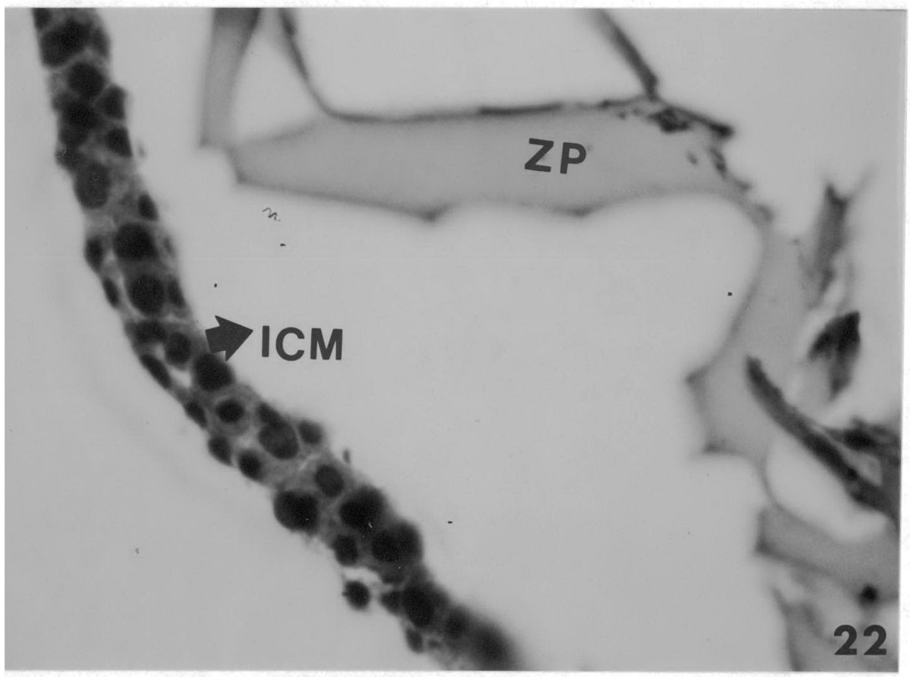

ZP
ICM
22

Fig 23: A section through the embryonic disc area of the rabbit blastocyst. Note the acidophilic (pinkish) cytoplasm and the basophilic (blue) nuclei. This section was stained with Harris' Hematoxylin stain. 160X

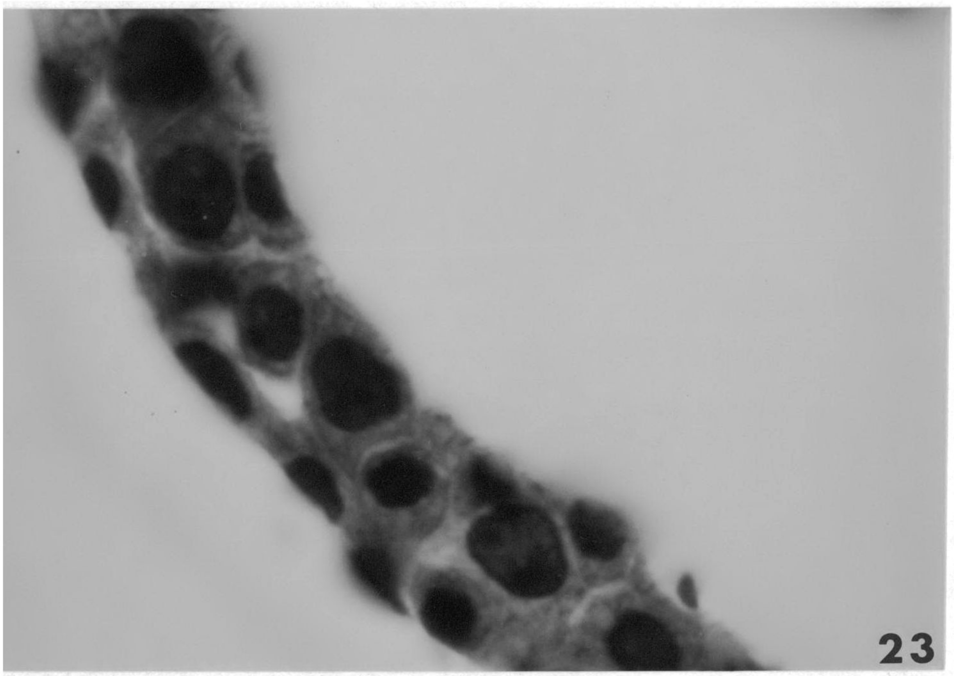

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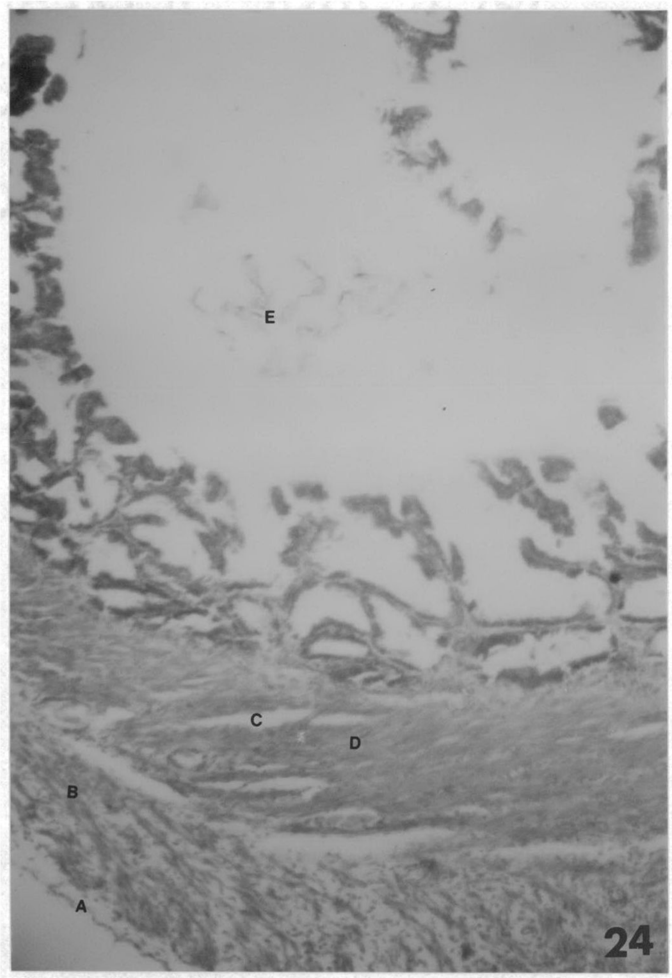
Fig 24: A section of a collapsed implanted 7-day-old rabbit blastocyst within the intact uterus. A. Uterine wall; B. Spiral arteries; C. Septum; D. Smooth muscle and E. Blastocyst. 25X


E
C
D
B
A
24

trace blastocyst development up to the point of formation of the heart. The cells that make up the inner cell mass of both the *in vivo* embryo and the *in vitro* cultured blastocyst, undergo rapid morphological changes to form other cell types, tissues, and organs in the embryo.

Examination of Fig 25 shows long branched striated fibers, with mononucleated cells in the 10-day-old embryonic heart. At this time the heart is not fully innervated (Dehaan, 1968).

F. In Vitro Observations

Figures 26, 27, and 28 show the rabbit 6 3/4-day blastocyst. Note the zona pellucida protecting the blastocyst which now consists of the trophoblast and the embryonic disc. It is clearly shown that the embryonic disc can be separated from the trophoblast with little alteration (Figures 29, 30, and 31). The embryonic disc, after 3 days in culture, developed pulsating cells. During this time some of the embryonic discs are attached and some are unattached. Pulsation occurred in the center of the embryonic mass (Fig 32). After four days in culture, pulsation occurs in the outer surface of the embryonic disc (Fig 33). Figures 34 and 35 show the unattached 6 3/4-day-old rabbit embryonic disc after five days in culture. Pulsation occurs in the convoluted bulbs which have developed.

G. Immunofluorescence

After observing the embryonic disc in culture, immunofluorescence techniques were carried out. The most widely used reagent that transfers fluorescent groups onto proteins is fluorescein isothiocyanate, Fig. 36. Figure 37, control, shows a bright field micro-

Fig 25: A section from 10-day-old rabbit embryonic heart stained with Harris' Hematoxylin. Note the striations in the heart (arrow). 160X

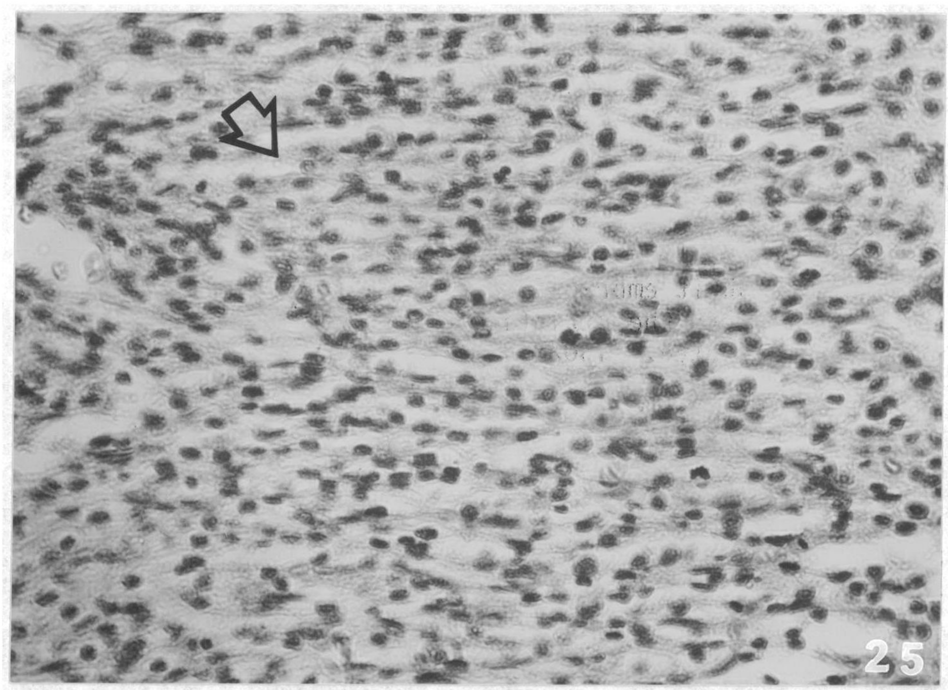

25

Fig 26: A light micrograph of the 6 3/4-day-old rabbit blastocyst. Note the zona pellucida (ZP), trophoblast (T) and embryonic disc (ED). 12.5X

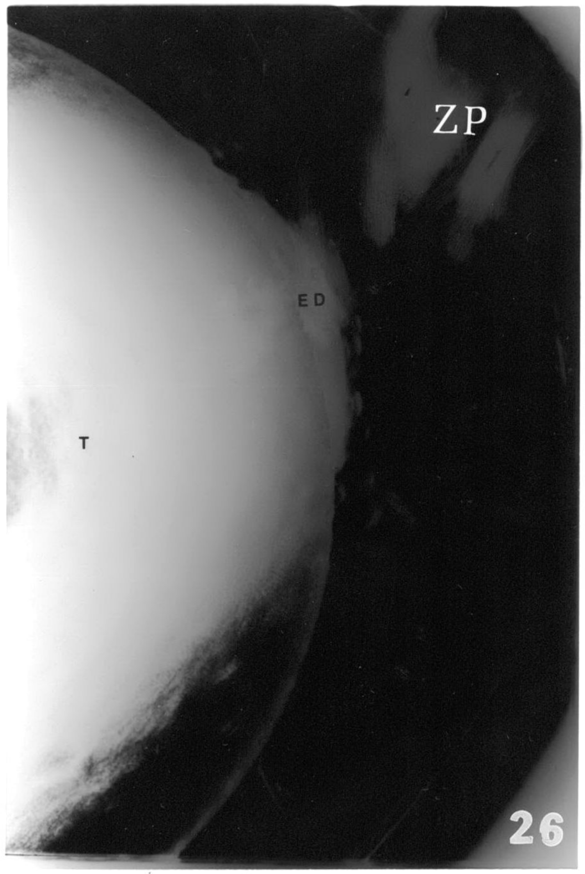

ZP
ED
T
26

Fig 27: A light micrograph of a 6 3/4-day-old rabbit blastocyst without the zona pellucida. Trophoblast (T) and embryonic disc (ED). 40X

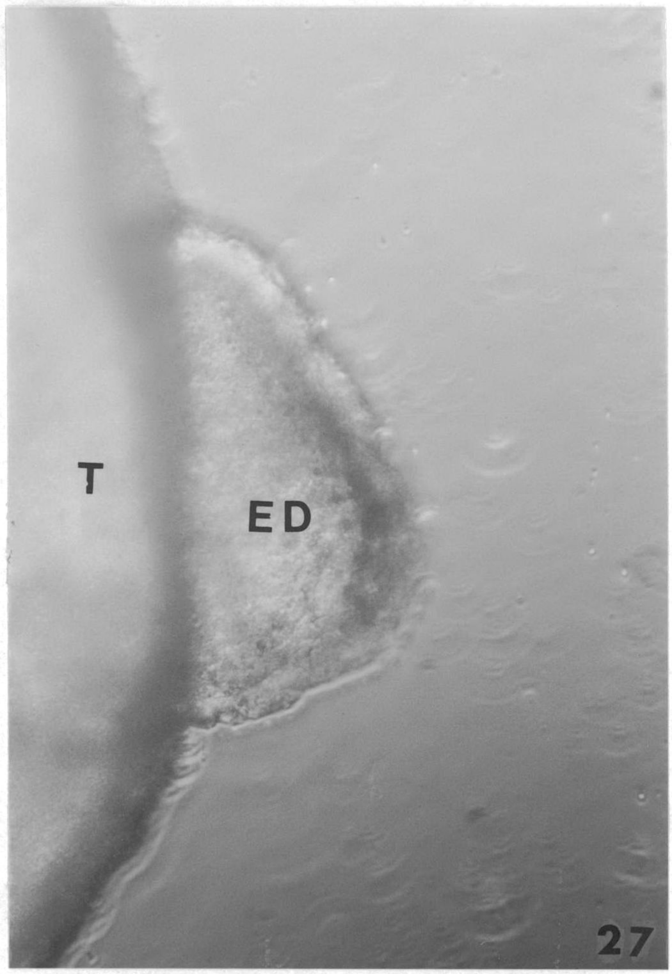

T
ED
27

Fig 28: The 6 3/4-day-old rabbit blastocyst with the embryonic disc oriented to appear on top. Trophoblast (T) and embryonic disc (ED). 40X

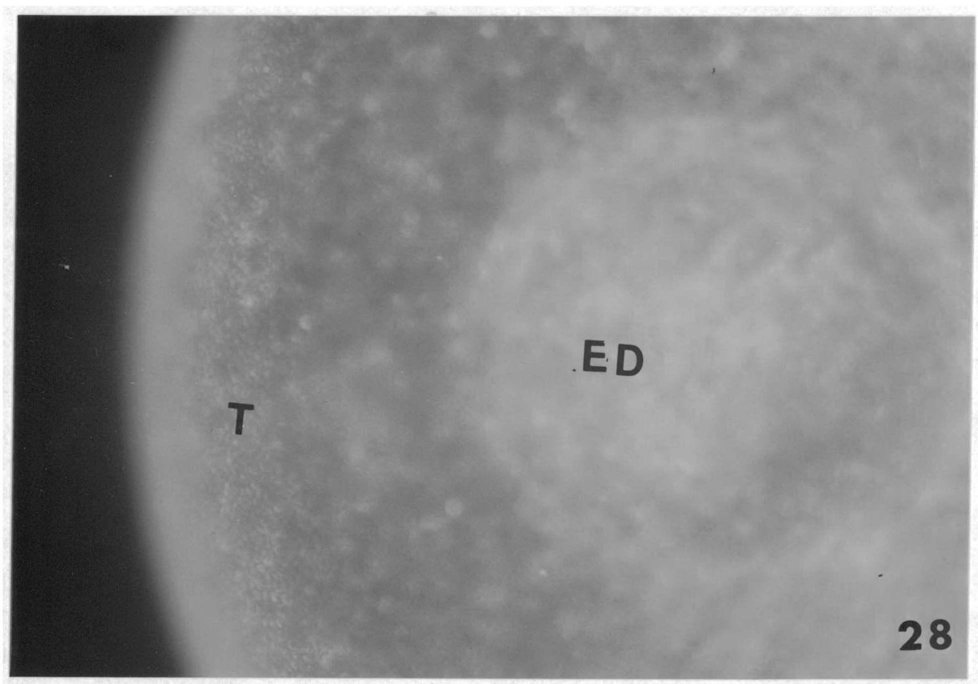

T
ED
28

Fig 29: The 6 3/4-day-old rabbit blastocyst after removal of the embryonic disc. Note the arrow indicates the detachment site of the embryonic disc from the trophoblast. 12X

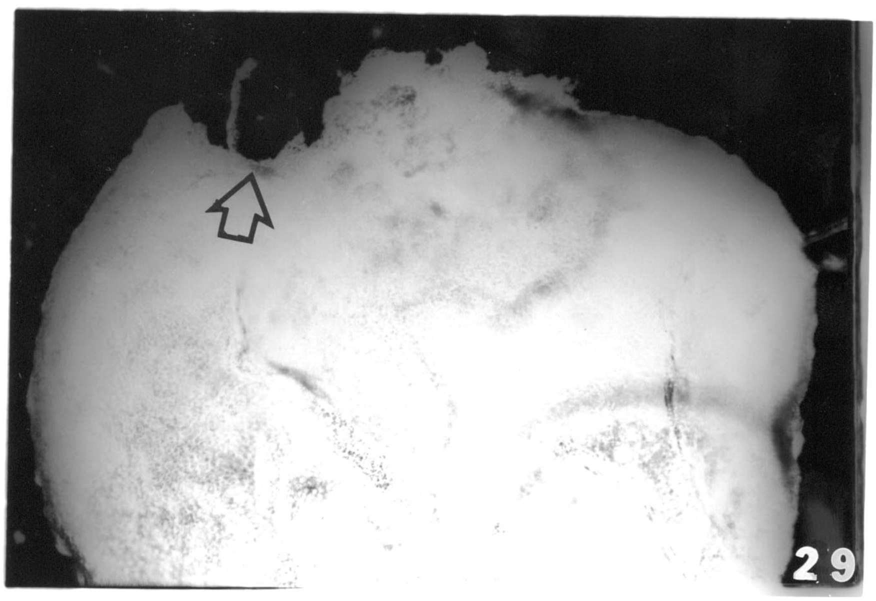


Fig 30: A light micrograph of the 6 3/4-day old rabbit trophoblast after removal of the embryonic disc. The trophoblast has reconstituted i.e. filled with fluid. The arrow indicates the site where the trophoblast has resealed. 12.5X

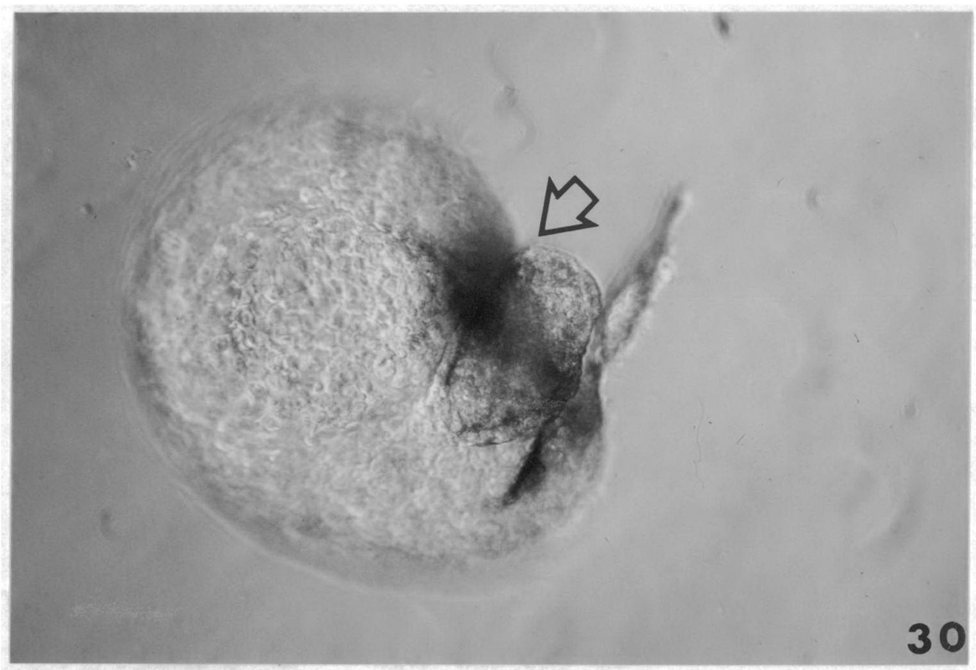
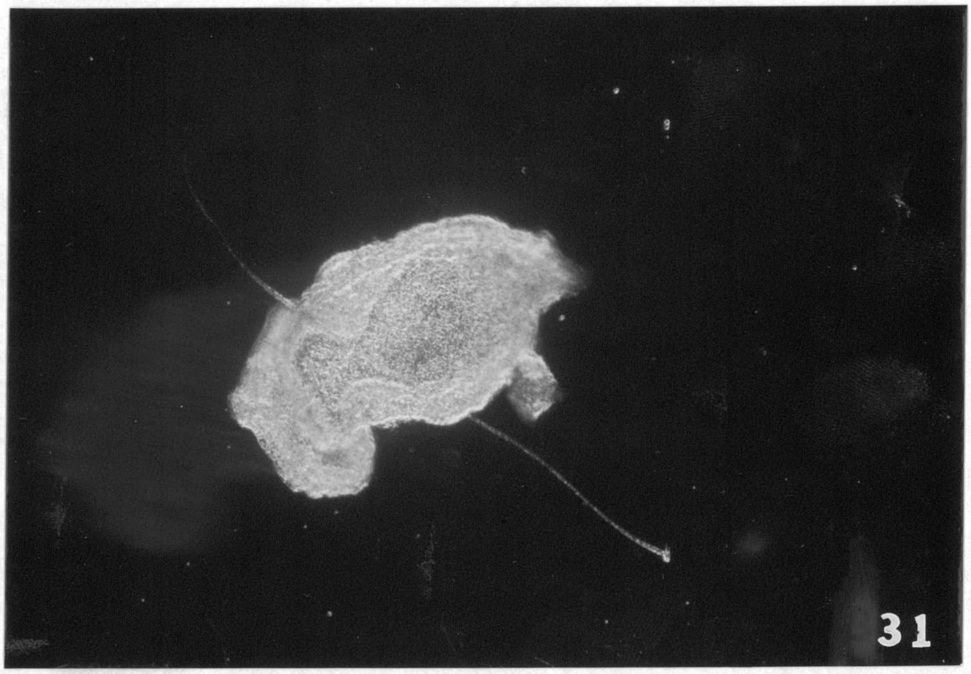

30

Fig 31: The 6 3/4-day-old rabbit embryonic disc after removal from the blastocyst. The embryonic disc remains unattached after two days in culture. 12.5X



31

Fig 32: An attached 6 3/4-day-old rabbit embryonic disc after three days in culture. Pulsation occurs in the center of the embryonic mass. Arrows indicate growth of fibroblasts (F). Pulsation occurs in dark-appearing area labeled (P). 40X

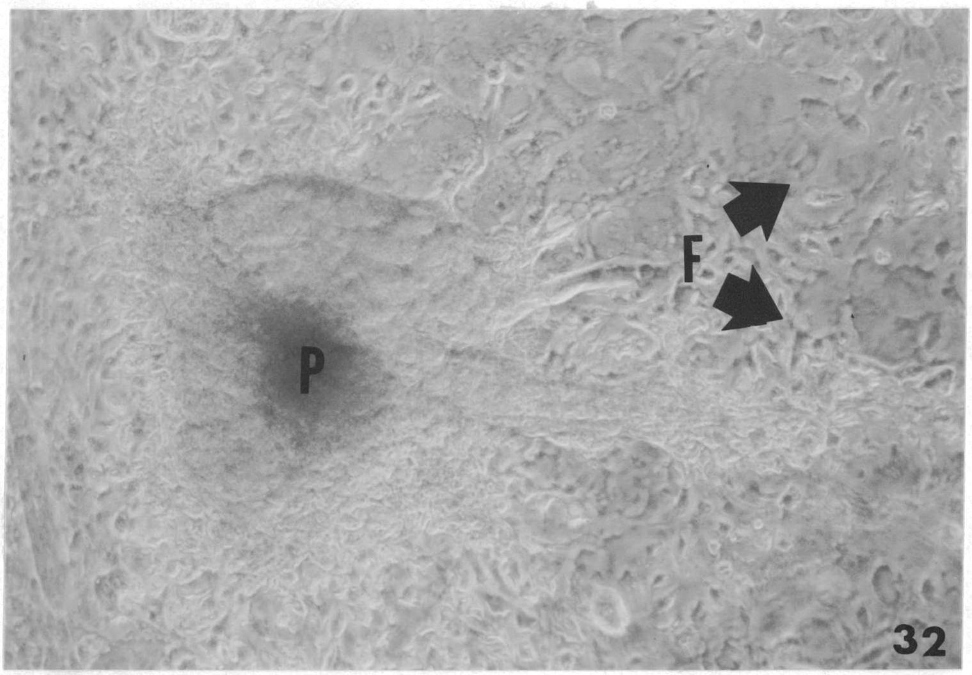

P
F
32

Fig 33: A light micrograph of the 6 3/4-day-old rabbit embryonic disc after removal from the blastocyst. The reconstituted embryonic disc remains unattached after four days in culture. Pulsation (P) occurs in the outer surface. 12.5X

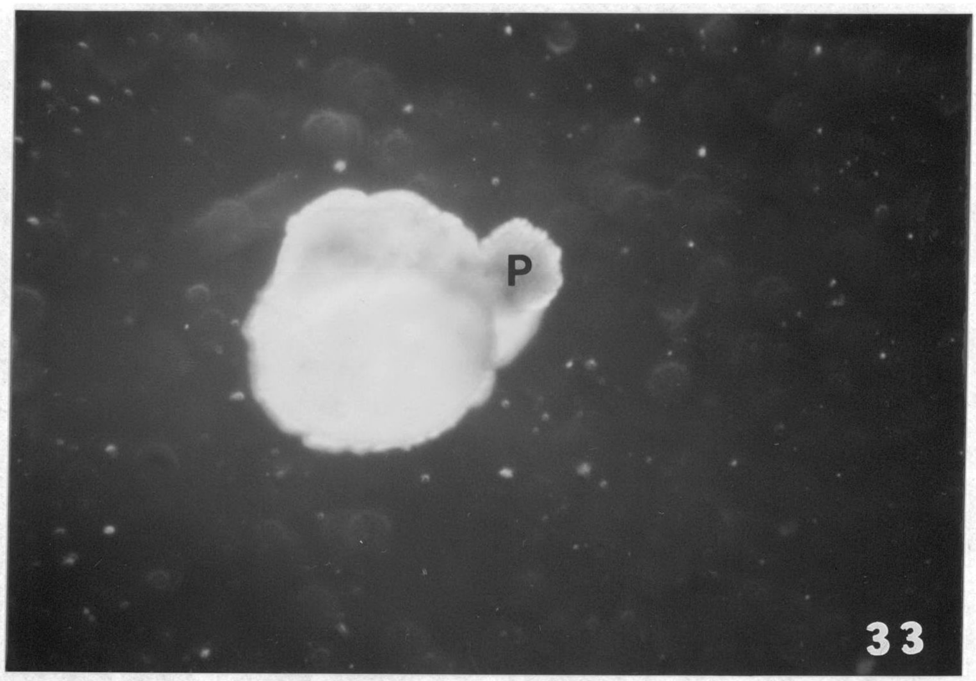
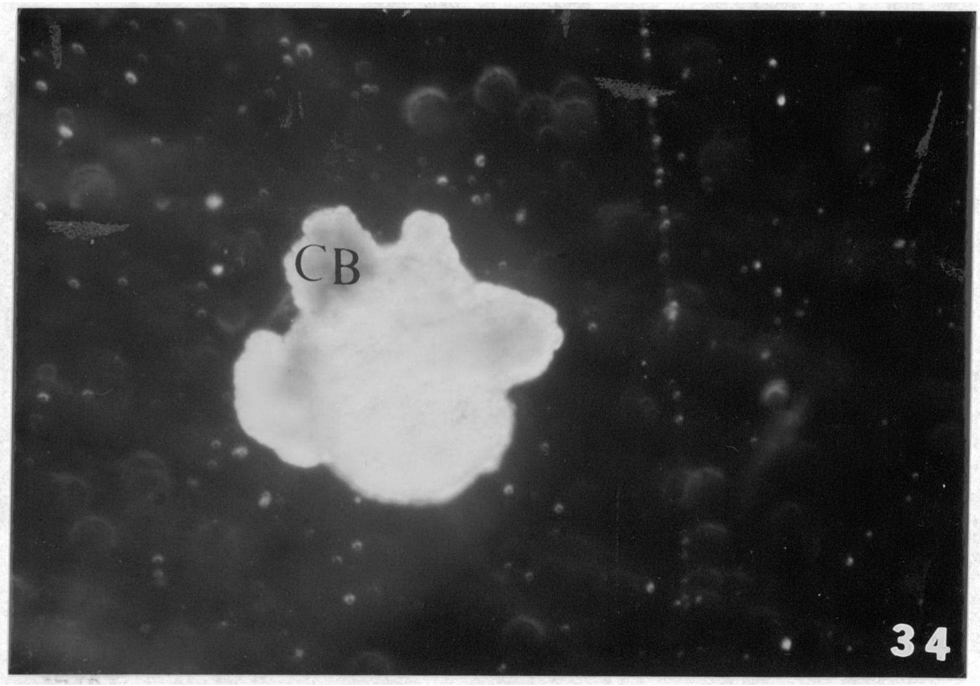

P
33

Fig 34: The 6 3/4-day-old rabbit embryonic disc after five days in culture. The embryonic disc still remains unattached. Pulsation occurs in the convoluted bulbs (CB). 12.5X

Fig 35: A higher magnification of the 6 3/4-day-old rabbit embryo after five days in culture. Arrow (CB) indicates area of pulsation in the convoluted bulb. 50X


CB
34

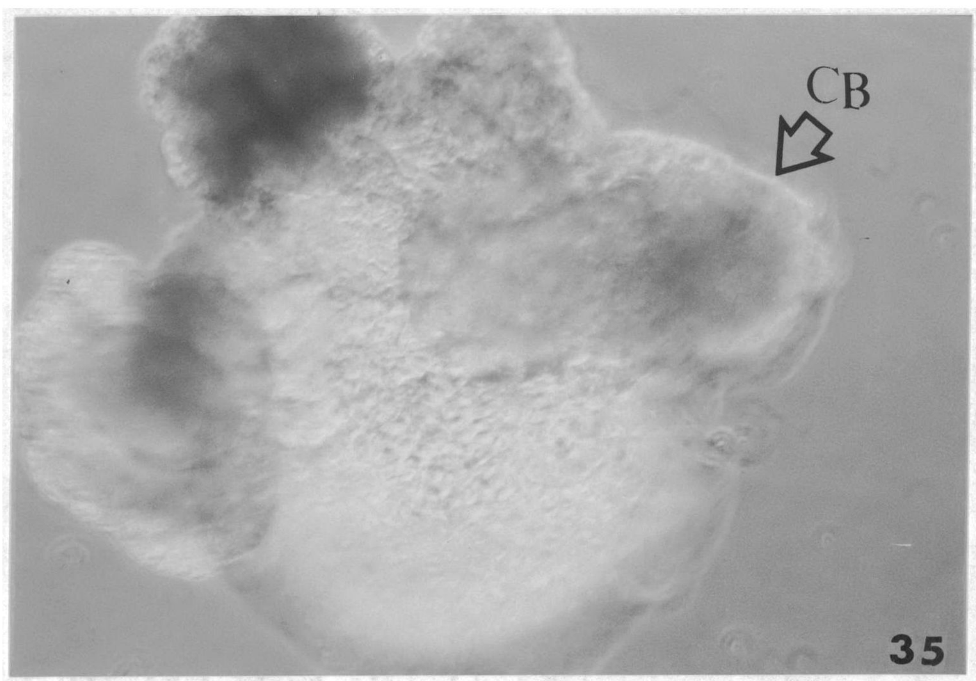
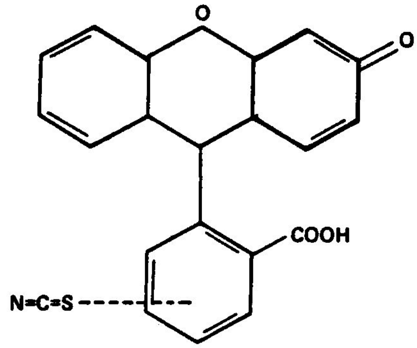

CB
35

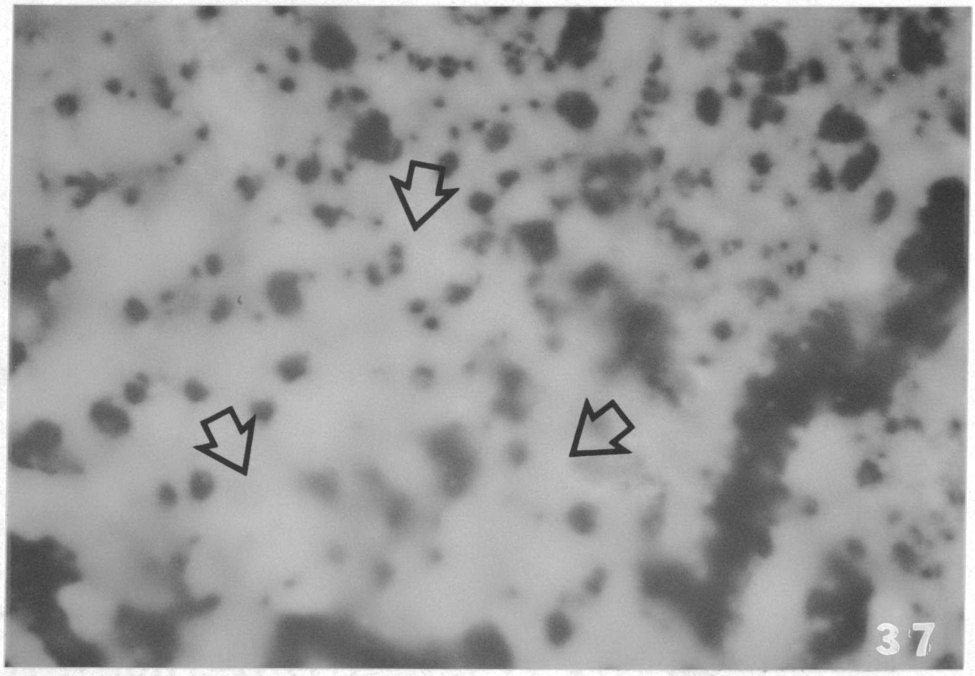
Figure 36: A coupling reaction of fluorescein isothiocyanate. The fluorescent dye, fluorescein isothiocyanate, can be coupled onto antibody molecules by allowing the isothiocyanate group to react with free amino-groups present on the antibody molecule (ab).



Fluorescein isothiocyanate (F^* – NCS)

Fluorescein - labeled antibody (F^* – AB)

Fig 37: A positive control showing bright fluorescence in a 10-day-old embryonic rabbit heart. The arrows indicate the sites of fluorescence. 128X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graph of a cryostat section of a 10-day-old embryonic rabbit heart done by the indirect fluorescent method as described in materials and methods. In comparision with a Hematoxylin-Eosin stained section (Fig 38) of a 10-day-old heart, it is clearly seen that cardiac myosin is present throughout the striated fibers. When examining the 10-day-old liver (data not shown) and the 6 3/4-day-old rabbit trophoblast (Fig 39) with anti-cardiac gamma globulin, no fluorescence was seen, suggesting that pre-cardiac cells are not present in these tissues. There was no fluorescence seen in the 5 3/4-day-old embryonic disc (Fig 40). Some indication of fluorescence was detected in the 6 and 6 3/4-day-old embryonic disc cells (Figs 41 and 42). Bright fluorescence was seen in the 6 3/4-day embryo after culture for 2 days (Fig 43) and 3 days (Fig 44). The intensity of fluorescence increased with time in the cultured embryonic discs.

Fig 38: A light micrograph of a 10-day-old embryonic heart stained with Hematoxylin-Eosin. The arrows indicate long branched striated fibers and mononucleated cells. 100X

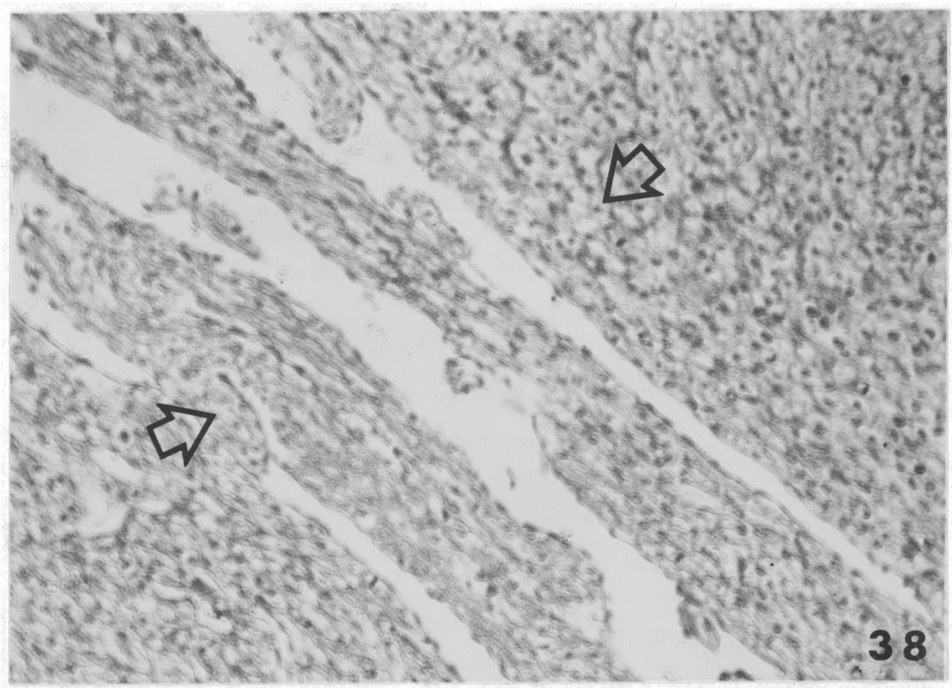
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Fig 39: A micrograph of the 6 3/4-day-old rabbit trophoblast treated with rabbit anti-cardiac myosin gamma globulins prepared in guinea pig. Note no fluoresence is seen. 16X

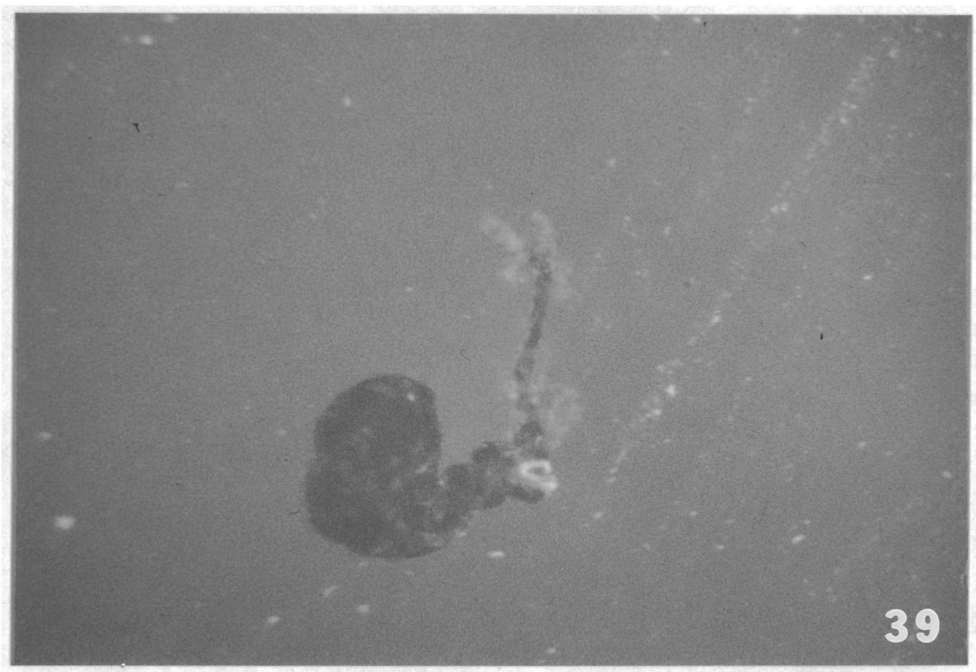
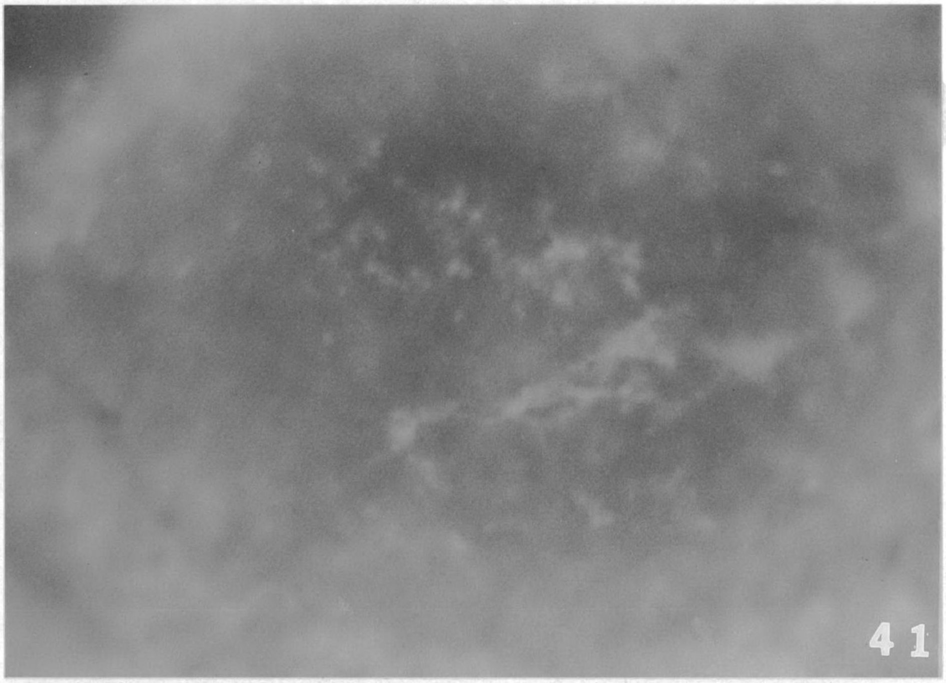

39

Fig 40: A fluorescence micrograph of the 5 3/4-day-old rabbit embryonic disc using the cover slip method to study localization of cardiac myosin. Note no fluorescence is seen. 80X


40

Fig 41: A cover slip fluorescence micrograph study of the 6-day-old rabbit embryonic disc. There is some indication of fluorescence at this stage. 80X

Fig 42: The 6 3/4-day-old unattached rabbit embryonic disc grown on cover slips for one day. Note fluorescence is more intense at this stage. 80X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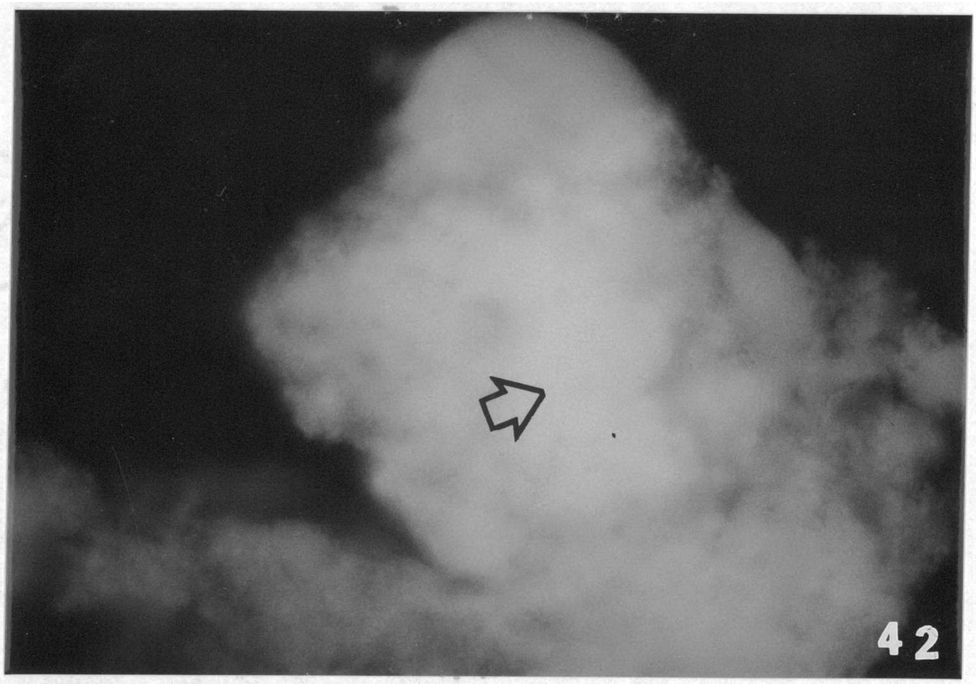
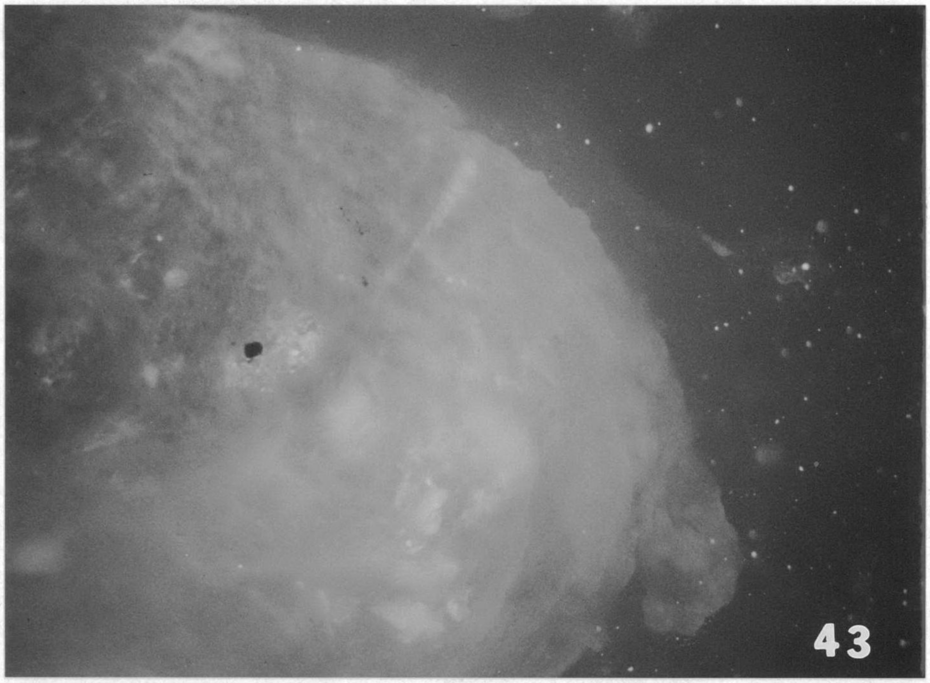
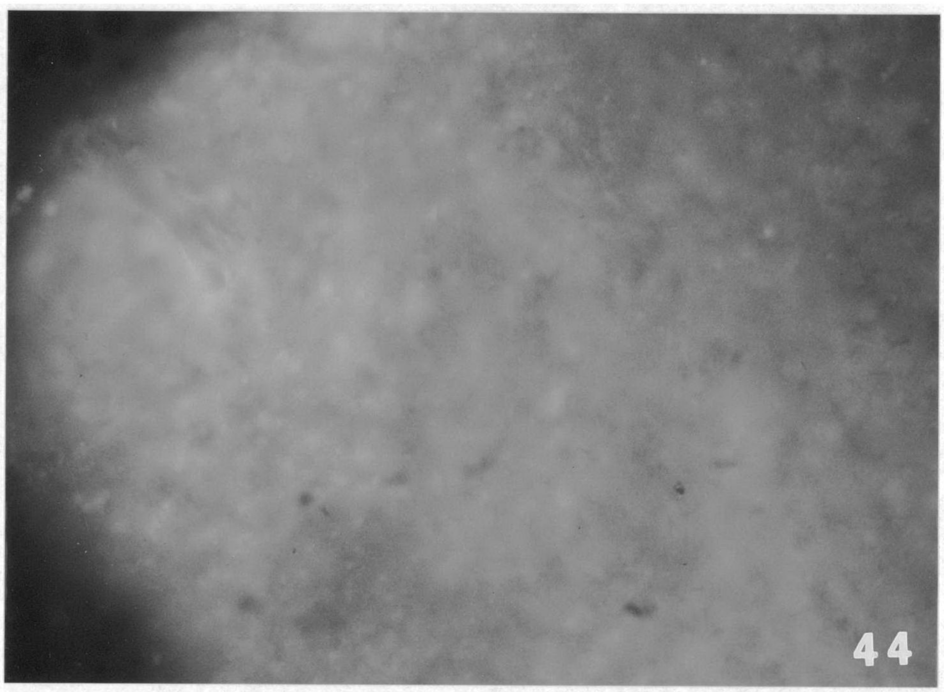

42

Fig 43: A fluorescence micrograph of the 6 3/4-day-old rabbit embryonic disc after two days in culture. Fluorescence is seen in more areas than in the one-day-old cultures. 80X.

Fig 44: A bright fluorescence micrograph of the 6 3/4-day-old rabbit embryonic disc after three days in culture. There is a greater distribution of fluorescence at this stage than in the above figure. 80X





CHAPTER V

DISCUSSION AND CONCLUSION

Contractile proteins play an important role in a number of processes. An understanding of the molecular mechanism of contraction requires the elucidation of the ontogeny, structure, biological function and localization of the contractile proteins.

The preparation of cardiac myosin samples, totally devoid of myosin aggregates, actomyosin, and associated constituents in muscle (such as C-protein, RNA and 5-adenylic deaminase), has been reported by Katz (1970) and Vieling et al. (1968) to be relatively difficult. The isolation procedures (Shiverick et al., 1974) employed in the present study appeared adequate in yielding consistent results from frozen and/or fresh tissue. In experiments where the molecular weight of myosin was determined by ultracentrifugation, several investigators have reported difficulty in obtaining pure myosin bands. Whether this was a reflection of the inherent tendency of myosin to aggregate (Katz, 1970) or resulted from the preparative methods (fresh and/or frozen starting material) could not be readily distinguished.

Measured values of the molecular weight of rabbit cardiac myosin (two heavy chains, approximately 200,000 D each and two light chains ranging from 25,000 D to 17, 500 D) although comparable, appeared to be consistently lower than corresponding values reported for myosin obtained from canine (Muller et al., 1964) and bovine (Tada et al., 1969) cardiac tissue. Two reasons may account for this observation: 1) differences in the preparative methods used and 2) species differences of the muscle source.

In addition to physiochemical studies of cardiac myosin, we examined rabbit cytoplasmic myosin extracted from adult rabbit liver. A myosin-like protein was identified in isolated rabbit liver cells. It was extracted with high ionic strength buffer containing ATP and subsequently purified. All attempts to purify hepatocyte myosin without protection from proteolysis (Brandon, 1976) resulted in at least partial degradation of the high molecular weight polypeptide. In this respect, the molecule shares, with skeletal muscle (Lowery, 1971) and platelet myosins (Adelstein, 1971), an extreme sensitivity to proteases. Myosin from polymorphonuclear leukocytes was partially degraded (Stossel and Pollard, 1973) under conditions similar to the ones used in our study. The effect of phenylmethylsulfonyl fluoride and sodium tetrathionate in stabilizing the myosin, suggests the involvement of serine and sulfhydryl protease. Assuming that the native molecular weight of hepatocyte (non-muscle) and skeletal muscle myosin isoenzymes are similar, and cardiac and skeletal myosin are similar, then the conclusion reached is that the hepatocyte myosin, like muscle proteins may be asymmetrical.

Myosin is widely distributed in non-muscle cells and it is expected that most cells containing actin will also have myosin. All myosins consist of both heavy and light chains, catalyze the hydrolysis of ATP, and interact with actin in ways which indicate that they could be the energy-transducing enzymes for cell mobility (Pollard and Weihing, 1974). In general, the myosin concentration is very low in nonmuscle cells. Our studies using polyacrylamide gel electrophoretic techniques indicate that hepatocyte (cytoplasmic) myosin molecular weights were comparable to other investigators, with heavy and light chains averaging HC-1 150,000 D, HC-2 111,000 D and LC-1 28,000 D and LC-2 19,000 D, respectively.

In contrast to the discrepancies with regard to the molecular weight of myosin, there seems to be a unanimous agreement concerning specific antigen/antibody reactions. Faced with these difficulties, several laboratories have turned to immunological techniques to localize myosin in non-muscle cells (Nachman et al., 1976; Groeschel-Stewart et al., 1970; Booyse et al., 1971 and Becker et al., 1973).

Ouchterlony is not an easy method to use with complex antigens, though the technique has its greatest benefit as an assay tool. Antigenic materials isolated by a variety of immunochemical and histochemical methods can be tested for purity and specificity in the agar gel test. In 1978 Yerna et al. produced antiserum against BHK_{21} (baby hamster kidney cells) myosin and discovered by immunodiffusion that it did not cross react with smooth or skeletal muscle myosins. These results confirm the reports published by Willingham et al. (1974) which demonstrate the lack of cross reactivity between a goat antibody directed against L-929 cell myosin and muscle myosin. Our analysis of adult cardiac myosin antigens and adult rabbit hepatocyte myosin antigens revealed homogenicity of antigen/antibody systems. When we crossed adult rabbit cardiac myosin antigen with anti-adult cardiac myosin antibody we got reactivity. The agar gel technique gave similar results using embryonic rabbit cardiac antigens and embryonic rabbit anti-cardiac antibody. When testing adult rabbit hepatocyte myosin antigen against adult anti-rabbit hepatocyte myosin antibody we again observed identity.

Examination of adult rabbit cardiac myosin antigen and adult rabbit anti-hepatocyte myosin antibody showed no cross reactivity. The same results were obtained when adult rabbit hepatocyte myosin antigen was reacted with rabbit anti-cardaic myosin antibody.

The combination of electrophoresis with precipitation in agar gel (immunoelectrophoresis) provided us with an additional method for identifying our antigen. Adult rabbit cardiac and adult rabbit cytoplasmic myosins were introduced separately into small wells in agar that had been cast on a plate, an ordinary microscope slide. After applying an electric field across the plate for 1 to 2 hours, the proteins are able to migrate, each according to its own electrophoretic mobility. After the proteins are separated, the electric gradient was discontinued and antisera (anti-cardiac and anti-cytoplasmic myosin, respectively) were introduced into wells which parallel the axis of the electrophoretic migration. The antibodies and antigens diffused towards each other forming a precipitation band, which indicates the intersection of their diffusion fronts. The immunoelectrophoresis experiment reveals the same results as our agar double diffusion experiments, identity with cardiac myosin and nonidentity with cytoplasmic myosin.

Antibody staining techniques have been used successfully in conjunction with fluorescence and electron microscopy to gain additional information about the localization of proteins. In order to localize a particular intracellular component by immunofluorescence, the antigen used to produce the antibody should be as pure as possible. In this study we used fluorescing antibodies to detect the appearance of cardiac myosin. The distribution of the fluorescence in our research agrees with experiments using actin antibody and fluorescein-labelled subfragment 1 (Goldman et al., 1975; and Schloss et al., 1977) showing actin-like microfilaments in cultured cells.

Other investigators have used immunofluorescence techniques to localize myosin within cultured cells. Weber and Groeschel-Stewart, 1974, used antiserum directed against chicken gizzard smooth muscle myosin to localize the distribution of myosin in 3T3 fibroblasts. The results of Willingham et al. (1974) coincide with our results using rabbit cardiac myosin. Our results show that pre-cardiac cells first appear in the 6-day-old embryonic disc. Increased fluorescence is seen in the 6 3/4-day-old embryonic disc cultured one, two, and three days indicating an increase in cardiac myosin with time. No indication of pre-cardiac cells was seen in the 5 3/4-day-old embryonic disc cells. In a recent study, Fujiware and Pollard (1976) have shown a distribution of myosin in the cytoplasm, cleavage furrow and mitotic spindle of human cells (HeLa cells). They also prepared antibodies against platelet myosin and HeLa cell extracts to localize myosin in the stress fibers of ENSON cells, another human cell line.

Separation of the two areas of the preimplantation blastocyst not only allows for detailed observations of the rabbit blastocyst, but can be compared with tissues that were histologically prepared. A comparison of living blastocysts in media and prepared blastocysts shows that the latter have irregularities in shape, size and structure, yet specific features are identifiable in each of these tissues.

One disappointment discovered while separating the embryonic disc from the trophoblast was the loss of orientation of the embryonic disc to the trophoblast as related to implantation. Implantation involves an initial attachment of the blastocyst to the surface of the luminal epithelium and this cellular contact could then allow an exchange of information from the blastocyst to the epithelial cells. Although the nature of such a signal is still unknown, it is thought to be a humoral substance released by an implanting blastocyst (Heald, 1976). It has been proposed that steroids synthesized or accumulated in the blastocyst may play an important role in implantation (Dickmann et al., 1976).

It is well known that in the rabbit, hamster, guinea-pig and monkey, normal implantation of the embryo can occur in the absence of the ovary provided progesterone is given exogeneously (Deanesly, 1960; Orsin and Meyer 1962; Meyer et al. 1969; and Kwun and Emmens, 1974). It has been suggested that in some of these species, the implantation site could be the source of estrogen which is synthesized or accumulated by the blastocyst (Singh and Booth, 1978).

In the uterus, a series of morphological and biochemical events occurs which leads to specific uterine cell death as the blastocyst attaches to and penetrates the uterine epithelial cell layer during implantation. It is believe that this is due to an involvement of lysosomal enzymes. In the localized cells, death could be initiated from within the cells by "programmed cell autolysis" or be introduced by factors released locally from the blastocyst (El-Shershaby and Hinchliffe, 1975 and Sengupta et al., 1979).

The mammalian blastocyst becomes enlarged by the accumulation of fluid within the blastocoel cavity. This swelling results from an active solute-linked water reabsorptive mechanism (Biggers et al., 1971). The rate of fluid accumulation during the implantation period shows a marked increase in cell number or blastocyst surface area (Borland et al. 1976). These increases in transport capacity are suggestive of differentiation or expression of different transport pathways. Morphological studies (Ducibella et al., 1975; and Hastings and Ender, 1975) of the rabbit embryo show that zonular tight junctions exist at the apical surface of the cells at the time of transition from the morula to the blastocyst stage.

Cole and Paul (1965) discussing the properties of cultured, preimplantation rabbit embryos, stated that they observed beating myocardial cells, blood islands and nerve cells. They mentioned that if the zona pellucida was removed with pronase, an embryo would form, the amniotic folds would close and the heart would develop. We observed that after the removal of the zona pellucida (Coleman, 1977) and cultivation of the embryonic disc for three to five days, pulsating cells appeared. Pulsation continued on an average of five to seven days. The rate of pulsation was on an average of 47 to 68 beats/min. There were characteristic differences in the shapes of cells from embryos with pulsating cells and those lacking pulsation. Some of the embryos lacking pulsating masses continued to grow, but not at a rate comparable to the pulsating embryos. Further, the cells were disorganized in embryos lacking pulsating masses. Growth and differentiation of embryos must proceed in an orderly fashion. This indicates the necessity for sequential induction if progressive development is to occur. Embryos which degenerate and lack any organized development do not proceed to the onset of pulsation.

During the early implantation stage in the rabbit, several events in the differentiation of the embryo are initiated. Once the zona pellucida has been removed, attachment occurs via the trophoblast cells. The inner cell mass (embryonic disc) comes to assume its mesometrial orientation, and undergoes changes leading to pulsating and/or non-pulsating cells. The *in vitro* culture studies of the pre-implantation stages of the rabbit blastocyst allowed a more direct observation and analysis of the embryonic disc.

It should be noted that the immunodiffusion and immunoelectrophoresis results allow us to conclude that guinea pig anti-rabbit cardiac myosin antibody is specific for cardiac cells and tissue. The quantitative immunoprecipitin technique used to measure the equivalence point is only suitable for measuring the optimal amount of antigen and antibody to use for maximum binding effect. Attempts to measure activity of cardiac and anti-cardiac myosin by immunoprecipitation on agar gel was unsuccessful. A series of immunofluorescence experiments demonstrated the localization of pre-cardiac cells.

The rabbit blastocyst is a useful model for studying cardiac myosin synthesis, however, development and differentiation could aid further studies dealing with regulation of cardiac myosin synthesis, which may possible contribute to dysfunctions and malfunctions related to myocardial disorders.

CHAPTER VI

SUMMARY

1. Adult rabbit cardiac myosin was isolated and characterized. The molecule was shown to have two identical heavy chains approximately 200,000 D each, a C-protein 40,000 D and two different light chains, LC-1 25,000 D and LC-2 17,500 D.

   Adult rabbit liver cytoplasmic myosin was also isolated and characterized. This molecule was shown to consist of two different polypeptide heavy chains, HC-1 150,000 D and HC-2, 111,000 D and two different light chains, LC-1 19,000 D and LC-2 16,000 D.

2. Antibodies were made against the isolated adult rabbit cardiac myosin and adult rabbit liver cytoplasmic myosin. Identity was seen by agar double diffusion and immunoelectrophoresis between cardiac myosin antigen and anti-cardiac myosin antibody, cytoplasmic myosin antigen and anti-cytoplasmic myosin antibody, but not between cardiac myosin antigen and anti-cytoplasmic myosin antibody nor cytoplasmic myosin antigen and anticardiac myosin antibody.

3. The embryonic discs were separated from the trophoblasts and placed in culture. Pulsating cells appeared within two days. Pulsation continued on an average of five to seven days. The rate of pulsation was an average of 47 to 68 beats/min.

4. Immunofluorescence studies showed distribution of cardiac myosin in cells of the embryonic disc of the 6-day-old blastocyst, but no fluorescence was observed in the 5 3/4-day-old embryonic disc.

The distribution of fluorescence increased with increasing age of the *in vivo* embryo or with days in culture for the *in vitro* cultured 6 3/4-day blastocyst.

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